

ACTIONS OF NEUROPEPTIDES ON MOUSE SPINAL NEURONES IN CULTURE

Peter William McCarthy

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1985

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ACKNOWLEDGEMENTS

I would like to thank the Scottish Hospitals Endowment Research Trust and S.E.R.C. for funding this project. I very much appreciate the time that Brian Powell spent preparing and maintaining the cultures. My fellow students and those postdoctoral researchers who have been on hand to answer the occasional questions have been a constant source of help, especially Tim Blaxter, Stuart Dunbar and Fiona Donald.

I would like to thank my supervisor Prof. G.A.Cottrell for all the time he has taken guiding me through this project and thesis.

DEDICATION

I should dedicate this thesis to my parents for their unwavering faith in me; my wife for her encouragement and my brother for his friendship and lunatic attitude towards life. Instead, I give this thesis to the memory of Mandy and Tanvier.

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SUMMARY

- 1] Spinal cords from mouse embryos were successfully prepared and maintained in primary dissociated cell culture, for periods in excess of 10 weeks.
- 2] Stable intracellular recordings were made from spinal neurones which had been sustained in these cultures.
- 3] Experiments were made on these spinal neurones using various amino acids and peptides. Solutions of these compounds were discretely applied by pressure ejection.
- 4] L-Glutamate, GABA and glycine evoked responses which appeared the same as those documented previously.
- 5] Ethylene-diamine did not evoke a response from the spinal neurones tested.
- 6] Only a small percentage of the spinal neurones responded to met⁵- and leu⁵- enkephalin, FMRFamide, neurotensin and glycyL L-glutamine. Supplementing the cultures with tissue from other organs did not increase the percentage of spinal neurones which were capable of responding to peptide.
- 7] Met⁵-enkephalin and leu⁵-enkephalin each evoked responses from the spinal neurones.
- 8] The enkephalin-evoked depolarizations accompanied by an increased input resistance were apparently voltage dependent. These responses were abolished at potentials more negative than -90mV and did not invert under normal recording conditions.
- 9] The enkephalin-evoked depolarizations associated with a decreased input resistance had extrapolated inversion potentials of -20mV. No voltage dependence was seen.

- 10] Enkephalins also evoked responses which had an inversion potential close to the resting membrane potential. These were accompanied by a decreased input resistance and were not desensitized by prolonged application of peptide.
- 11] None of these responses showed obvious desensitization with prolonged application, however, they were all attenuated by naloxone.
- 12] Met⁵-enkephalin was apparently more potent than leu⁵-enkephalin on a small number of neurones. Furthermore, met⁵-enkephalin application, during the weaker response from those neurones to leu⁵-enkephalin, evoked a attenuated response.
- 13] FMRFamide evoked two responses from these spinal neurones. These responses were seen separately and mixed. In the latter case they were referred to as biphasic responses.
- 14] The depolarizing response to FMRFamide was accompanied by an increase in input resistance. Potassium had some involvement in these responses.
- 15] The FMRFamide responses which were accompanied by a decreased input resistance showed a great variety of inversion potentials between neurones. These actions were dependent upon sodium and chloride ions.
- 16] Enkephalin and FMRFamide, when applied separately to the same spinal neurone, did not evoke the same response.
- 17] Responses evoked by neurotensin were hyperpolarizations associated with a decreased input resistance. These responses were dependent upon potassium and independent of chloride ions.

- 18] Glycyl L-glutamine evoked two types of hyperpolarizing response from the spinal neurones. These could appear separately or combined.
- 19] The faster responses to glycyl L-glutamine were apparently dependent on potassium ions.
- 20] The slower responses to glycyl L-glutamine were apparently insensitive to changes in extracellular potassium or chloride. However, these responses were sensitive to intracellular injection of chloride ions.
- 21] At concentrations of peptide which evoked a response from other spinal neurones, none of the peptides produced any measurable modulation of amino acid-evoked responses.

ABBREVIATIONS

DALA	D-ala ² - leu ⁵ -enkephalinamide
DADLE	D-ala ² - D-leu ⁵ -enkephalin
DAME	D-ala ² - met ⁵ -enkephalin
DRG	Dorsal root ganglion
e.p.s.p.	Excitatory post-synaptic potential
GABA	Gamma-amino butyric acid
Hepes	N-2-Hydroxyethylpiperazine- N'-2-ethanesulphonic acid
i.p.s.p.	Inhibitory post-synaptic potential
msec	milliseconds
mV	millivolts
nm	nanometre
sec	seconds
TTX	Tetrodotoxin
um	micrometre

AMINO ACIDS: SINGLE LETTER ABBREVIATIONS

As used for the smaller peptides [see IUPAC-IUB, 1972].

F	Phenylalanine
G	Glycine
M	Methionine
L	Leucine
R	Arginine
Y	Tyrosine

As an example; FMRF is the equivalent of Phe-Met-Arg-Phe.

CHAPTER I

INTRODUCTION

Numerous peptides have been found in the mammalian central nervous system [CNS]. These peptides have been shown, using immunohistochemical methods, to be specifically located in neuronal pathways in the CNS [Hökfelt, Johansson, Ljungdahl, Lundberg and Schultzberg, 1980a; Hökfelt, Lundberg, Schultzberg, Johansson, Skirboll, Anggard, Fredholm, Hamberger, Pernow, Rehfeld and Goldstein, 1980b]. Further to this, studies of brain preparations in vitro have shown that certain peptides can be released into the surrounding medium [Iversen, Lee, Gilbert, Hunt and Emson, 1980]. This release could be evoked by high potassium ion concentrations in the perfusing medium, provided that calcium ions were present also. This evidence, in addition to reports of peptide release from nerve tissue [Otsuka and Konishi, 1976; Yaksh, Jessell, Gamse, Mudge and Leeman, 1980; Rokaeus, Fried and Lundberg, 1982], suggests that certain peptides can be released from neurones during neurotransmission. Furthermore, the ability of certain peptides to alter spontaneous neuronal activity [Phillis and Kirkpatrick, 1980] implies that they have a role as neurotransmitters and/or neuromodulators in the mammalian CNS.

Peptides which have been localized within the nervous system have become known as neuropeptides. At present there are almost 50 members of the class neuropeptide. These range from dipeptides [carnosine and glycyl L-glutamine] to large polypeptides; such as adreno-corticotrophic hormone [ACTH], pancreatic polypeptide, cholecystokinin and β -endorphin, [Iversen, 1983; Mutt, 1983]. However, there still remains speculation concerning the possible role of neuropeptides in inter-neuronal communication.

Just how do the neuropeptides compare with the "classical" neurotransmitters such as noradrenaline and acetylcholine? It appears as though there are both similarities and differences between these two groups. Neuropeptides have been located in nerve terminals [Elde, Hökfelt, Johansson and Terenius, 1977; Cuello and Kanazawa, 1978] and have been shown to be released on nerve stimulation [Otsuka and Konishi, 1976; Yaksh et al., 1980; Rokaeus et al., 1982]. In addition, neuropeptide release can be caused by increasing the potassium ion concentration in the presence of calcium [Iversen, Iversen, Bloom, Vargo and Guillemin, 1978; Mudge, Leeman and Fischbach, 1979; Iversen et al., 1980]. Neuropeptides can also alter spontaneous neuronal activity [Phillis and Kirkpatrick, 1980]. Thus, the gross actions of the neuropeptides appear similar to those of the "classical" neurotransmitters [Schousboe, 1982].

The mechanisms by which each of the systems function, however, appear to be different. The "classical" neurotransmitters can be recycled at the nerve terminal, whereas: 1] there is as yet no evidence for a neuropeptide re-uptake mechanism [Jessell, Iversen and Kanazawa, 1976; Jessell, 1983]; instead they appear to be broken down by a variety of enzymes [Schwartz, 1983] and, unlike acetylcholine, the breakdown products are not necessarily re-used by the neurone, 2] the neuropeptides are only synthesized as part of large polypeptides, in the cell soma [Habener, 1981]. This has suggested quite strongly that the neuropeptides would be used only once and are not recycled by the neurone. There may also be differences in the way neuropeptides produce responses from neurones [e.g., the modulation of amino acid responses and effects on action potential threshold as described by Barker, Gruol, Huang, Neale and

Smith, 1978a]. From this, it would appear that a study of the neuronal responses evoked by neuropeptides would give a greater insight into the functioning of inter-neuronal communication in the CNS.

Five peptides were chosen from the large number of neuropeptides presently available. Initially, only met⁵- and leu⁵- enkephalins were tested. However, because of the wide variation in sensitivity of the cultured neurones to these peptides [this aspect will be discussed in chapter VI], other peptides were also included. These were neurotensin, Phe-Met-Arg-Phe-amide and glycyl L-glutamine. For clarity, each of these peptides will be dealt with separately.

I.1] ENKEPHALINS

I.1.1] ISOLATION AND CHARACTERIZATION

The first successful isolation and characterization of endogenous opiates was by Hughes, Smith, Kosterlitz, Fothergill, Morgan and Morris, [1975]. These workers reported the isolation of two similar pentapeptides from extracts of porcine brain. These peptides had the amino acid sequences Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu; YGGFM and YGGFL respectively [using single letter abbreviations, IUPAC-IUB, 1972] and were named enkephalins. The name of the C-terminal amino acid was used as a prefix to allow discrimination between them e.g., methionine⁵- or met⁵- enkephalin and leucine⁵- or leu⁵- enkephalin. Both of these enkephalins showed opiate-like activity when tested on in vitro

preparations of either mouse vas deferens or guinea pig ileum , [Hughes et al., 1975]. Confirmation of the structures deduced by Hughes and co-workers, [1975] came with a report by Simantov and Snyder [1976] who isolated the same two opioid pentapeptides from bovine brain. At present, there are many different opioid peptides [Bloom, 1983]. However, this thesis will be mainly concerned with just two; met⁵- and leu⁵- enkephalins.

I.2] PHARMACOLOGY OF ENKEPHALINS IN THE MAMMALIAN CNS

The following sections describe the variety of responses which have been evoked by various enkephalins in studies of neurones in the mammalian CNS. The following sections serve to illustrate two facts: the diversity of the responses to the opioids; and how the complexity of the CNS can hamper investigations into the mechanisms underlying neuronal responses. Throughout the thesis, the word opioid will refer to peptides with opiate-agonist activity and opiate will be used to refer to the alkaloids [e.g., morphine].

I.2.1] CEREBRAL CORTEX

A number of reports have described the effects of enkephalins in the cerebral cortex in vivo. Application of met⁵-enkephalin into the rat cerebral cortex caused both increases and decreases in the spontaneous activity of various neurones. In their extracellular studies, Hill, Pepper and Mitchell [1976a] and Hill, Mitchell and Pepper [1976b], showed that the majority of opioid peptide responses were depressions of activity [59 of 88 neurones]. However, in 2 neurones enkephalin evoked an increase in activity. All of these

responses were of rapid onset and quickly reversed following the cessation of peptide application. These effects were not desensitized by repeated peptide application. The depression of neuronal activity was attenuated by naloxone in only 2 of 14 experiments and neither of the excitatory actions were tested with an opiate antagonist.

Discrimination between the frontal and posterior cerebral cortices, with respect to enkephalin responses, came with a report by Frederickson and Norris [1976]. These workers showed that the enkephalins could only affect activity in the frontal cerebral cortex of the rat and not the posterior cortex. The majority of responses evoked by met⁵-enkephalin were inhibitory: 34 of the 63 neurones tested with enkephalin responded with a reduction of both the spontaneous and the L-glutamate-evoked neuronal activity. A further 5 of those 63 neurones responded to enkephalin with an increase in activity. The inhibitions of activity evoked by enkephalin were attenuated by naloxone, [in 11 of 14 tests]. However, in common with the previous study, the effects of naloxone were not tested with respect to the excitatory responses. In contrast, an extracellular study of the rat cerebral cortex by Davies and Dray, [1978] described the majority of opioid-evoked effects as being excitatory.

The enkephalins evoked biphasic responses which consisted of an inhibition of neuronal activity followed by an excitation, Davies and Dray [1978]. These biphasic responses were comparable with those reported by Bradley, Briggs, Gayton and Lambert, [1976] as well as those of Gent and Wolstencroft, [1976] in the brainstem, and Hill, Mitchell and Pepper, [1977] in the hippocampus. Both the excitatory

and the inhibitory responses were reversibly attenuated by naloxone [Davies and Dray, 1978]. Met⁵- and leu⁵-enkephalin were tested in the latter study. However, insufficient neurones were tested with leu⁵-enkephalin to allow any accurate comparison of their individual effects. In confirmation of the latter report, enkephalin depressed 2 and excited 9 of the 18 neurones which were tested in the rat cerebral cortex, [Stone, 1983]. Naloxone reversed both of the depressant effects, but only attenuated 1 of the 4 excitatory effects evoked by met⁵-enkephalin.

These responses were all studied at the extracellular level. This was because of the difficulties of recording with an intracellular electrode from an in vivo preparation added to the low density of opiate receptors present in this brain region [Pert, Kuhar and Snyder, 1976]. In the following section, the problem of access was overcome by making a slice of the brain which contained the relevant brain area. However, again the density of the opi receptors was low [Pert et al., 1976].

I.2.2] HIPPOCAMPUS

Acetylcholine and L-glutamate excited all of the neurones tested in the rat hippocampus, in vivo. In the same study, approximately 40% of the neurones were excited by an opioid [Hill et al., 1977]. The opioids were met⁵- or leu⁵-enkephalin, BW180C and D-ala²-D-leu⁵-enkephalin [DADLE]. Another 40% of the neurones had their spontaneous or L-glutamate-evoked activity depressed by opioid. A small number of neurones exhibited biphasic responses which were comparable to those found in brainstem [Gent and Wolstencroft, 1976]

and in cerebral cortex [Davies and Dray, 1978]; the response being a reduction followed by an increase in activity.

By electrically stimulating the alveus or stratum radiatum of the rat hippocampal slice preparation the pyramidal cells could be activated either anti- or ortho-dromically. In the presence of D-ala²-leu⁵-enkephalinamide [DALA] the initial population spike, caused by the orthodromic stimulation, was larger and the number of population spikes was increased, [Nicol, Alger and Jahr, 1980]. There was no change in the amplitude of the antidromic response. The effect on the orthodromic response was attenuated or even reversed in the presence of naloxone. These results suggested that the opioids enhanced the excitatory synaptic responses of the pyramidal cells. An increase in the spontaneous activity of hippocampal neurones in response to enkephalin was also described by Nicol, Siggins, Ling, Bloom and Guillemin, [1977]. In the latter study the opioid responses were attenuated by naloxone which was applied by either iontophoresis or subcutaneous injection.

In a study of 150 pyramidal cells, 128 were excited, 8 were depressed and the remaining 14 were unaffected by met⁵-enkephalin. In this study by Zieglansberger, French, Siggins and Bloom [1979], the activity from non-pyramidal cells positioned close to the hippocampus was shown to be depressed by the opioid. These workers reported that prior administration of magnesium ions to the area around the pyramidal cells attenuated the opioid-evoked increases in spontaneous activity, [25 of 35 tests]. This treatment did not affect the increased activity produced by the application of acetylcholine. The presence of bicuculline in the recording medium

also antagonized the neuronal excitation evoked by opioid peptides. However, the spontaneous activity of the neurones was increased by both the high magnesium and the bicuculline. These increases in spontaneous activity may have swamped the enkephalin responses.

Simultaneous extracellular recordings have been made from pairs of loci in the hippocampus, between 30 and 180um apart, [Zieglgansberger et al., 1979]. This enabled a study of activity from 2 closely positioned cells, e.g., a pyramidal cell and an interneurone. These workers found increases in pyramidal cell activity which were synchronous with depressions of activity in the nearby cells [interneurones] during opioid application, suggesting that the increases in pyramidal cell activity were caused by disinhibition of a tonic inhibitory input.

An intracellular study of the pyramidal cells showed DALA to have little effect on either membrane potential or membrane input resistance, [Nicoll et al., 1980]. However, the inhibitory post-synaptic potential [i.p.s.p.] evoked by antidromic [alveus] stimulation was found to be attenuated by DALA, as was the late slow excitatory post-synaptic potential [e.p.s.p.] evoked by orthodromic [stratum radiatum] stimulation. The presence of opioid also blocked spontaneous i.p.s.p.s in hippocampal pyramidal cells. All of these effects were reversed by naloxone. The opioids failed to have any effect on the direct cellular response of the pyramidal cells to GABA. Taken together, the results of this study suggested that the opioids caused disinhibition by a reduction of transmitter release from an inhibitory interneurone.

The suggestion of disinhibition by the opioids was questioned in a report on the CA1 region of the rat hippocampal slice. The responses obtained from extracellular studies of evoked activity were similar to those of Nicoll et al., [1977,1980]. However, in this study, Haas and Ryall [1980] reported an increase in the e.p.s.p. amplitude in CA1, CA3 and dentate granule neurones but no decrease in the amount of inhibitory activity during the presence of opioid. It was shown in further intracellular studies that the e.p.s.p. was not accompanied by any marked change in membrane potential or input resistance. Tachyphylaxis of these opioid effects was suspected during opioid application from multibarrelled pipettes. This led to the questioning of data obtained in other studies in which opioids were applied by either superfusion [e.g., Nicoll et al., 1980] or iontophoresis from multibarrelled pipettes [e.g., Zieglgansberger et al., 1979]. Haas and Ryall [1980] concluded that the action of opioids in the rat hippocampus was to increase the release of an excitatory neurotransmitter from a neurone presynaptic to the pyramidal cells.

Tachyphylaxis was also reported for opioid responses evoked from neurones in the cultured hippocampal slice. Gahwiler [1980] reported that large long-lasting e.p.s.p.s, often with superimposed action potentials, accompanied prolonged bath application of opioid. These depolarizing events [cf. Barker et al., 1978a] were abolished by either tetrodotoxin or high concentrations of magnesium ions in the extracellular medium. The opioids also caused a large reduction in the amplitude of the bicuculline-sensitive i.p.s.p.s which had been evoked by field stimulation, [Gahwiler, 1980]. However, in most pyramidal cells the only effects were increases in amplitude and

duration of evoked e.p.s.p.s. The conclusions from this study were that the opioid action was a disinhibition by a decrease in the amount of inhibitory transmitter released.

In a more recent extracellular study, low concentrations of DADLE [10nM] were applied to the rat hippocampus by superfusion. Lynch, Jensen, McGaugh, Davila and Oliver, [1981] found no change in the amplitude of e.p.s.p.s recorded from apical dendrites during the presence of opioid. Likewise, opioid application did not reduce feedforward or feedback inhibition at concentrations which increased the population spike. The authors suggested that opioid evoked increases in the population spike were mediated by an increased coupling of the dendritic e.p.s.p. to a mechanism for the generation of action potentials in the soma.

The possibility of an increased membrane input resistance, or an alteration in the threshold for action potential generation, was the subject of an intracellular study of the guinea-pig hippocampal slice. Masukawa and Prince [1982] reported no changes in either of those parameters, nor any alteration in the after-hyperpolarization which followed the action potentials, in pyramidal cells from the CA1 and CA3 regions. DADLE did evoke a small change in membrane potential. This latter effect resembled that reported by Nicoll et al., [1977] which was apparently caused by a reduction in the number or size of spontaneous i.p.s.p.s. However, both of these studies used superfused opioid and were thus liable to desensitize [Haas and Ryall, 1980; Gahwiler, 1980] which may have masked part of the response.

The overall picture for this region of brain is confusing. This is because of the conflicting results [cf. Nicoll *et al.*, 1977,1980; Haas and Ryall, 1980; Lynch *et al.*, 1981]. These conflicts apparently stem from the variations in methods used to obtain the responses and possibly even the treatment of the tissue [cf. Gahwiler, 1980]. Except for Masukawa and Prince [1982], all of these studies were on the rat hippocampus. The bulk of the evidence supports the disinhibition hypothesis. However, confirmation of this will have to wait until enkephalin responses have been studied in interneurons.

Another possibility for these anomalous results was highlighted when a variety of opioids were tested on hippocampal neurones. With the exception of ethylketocyclazocine, all of the opioids tested evoked excitations which were similar to those already mentioned, [Bradley and Brookes, 1981]. In this study ethylketocyclazocine produced a depression in activity. All of these opioid-evoked responses from the hippocampal neurones were attenuated by naloxone. The ability of the various opioids to evoke effects from these neurones introduces the question of a role for different opioid receptor subtypes in these responses. The work of Dingledine, Valentino, Bostock, King and Chang, [1983] and Bostock, Dingledine, Xu and Chang, [1984] illustrates this possibility. These workers have shown that the opioid effects in the rat hippocampus are mediated by at least the Mu- and Delta- sub-types of receptor. These studies were of CA1 pyramidal neurones and used extracellular recording techniques alongside ligand binding studies. If the opioids show similar overall effects with slightly different

underlying mechanisms, as with DRG neurones in culture [cf. Werz and MacDonald, 1984] then this could explain the controversy between the various reports from the hippocampus.

In the hippocampus a major set-back to the research was the inability of the workers to make reliable intracellular recordings from the interneurons around the pyramidal cell layer. This has led to the use of many subtly different, but indirect, methods of obtaining the data which in turn has led to the controversy. In the next section, the mixture of a higher density of opioid receptors [Pert et al., 1976] and a preparation which allowed access to the cells were employed in a detailed study of an opioid response.

I.2.3] LOCUS COERULEUS

A depression of neuronal activity was produced following enkephalin application to spontaneously active neurones of the rat locus coeruleus in vivo [Guyenet and Aghajanian, 1977]. This effect was of rapid onset and fully recovered following the termination of enkephalin application. Young, Bird and Kuhar [1977] used a larger sample size than Guyenet and Aghajanian, [1977] in a further extracellular study of this brain region [76 neurones as opposed to 8]. The larger sample size enabled a study of the area around the locus coeruleus for sites of opioid peptide action. They showed that the majority of enkephalin responses were situated in or near the nucleus locus coeruleus and the parabrachial nucleus, and also confirmed that enkephalin produced a reduction in the spontaneous neuronal activity. These opioid responses had a rapid onset and recovery and were attenuated in the presence of naloxone.

Following these extracellular studies in the rat by Young et al., [1977] and Guyenet and Aghajanian [1977], the enkephalin responses of neurones in the locus coeruleus were studied in the guinea-pig. However, in these studies the nucleus locus coeruleus was maintained in vitro; in a slice of the guinea-pig brain. This made possible an intracellular study of the neurones in the locus coeruleus and their responses to opiates, [Pepper and Henderson, 1980]. In this study, normorphine [100nM to 3uM] was applied by superfusion to 28 neurones. Of those; 19 were hyperpolarized, 1 was depolarized and the remaining 8 were unaffected by the opiate. These opioid-evoked hyperpolarizations were accompanied by a decrease in membrane input resistance. Pepper and Henderson [1980] also reported that the hyperpolarizing response was concentration dependent. It had an onset within one minute of the start of perfusion and was easily reversible on washing. A marked reduction and even abolition of any accompanying spontaneous activity occurred during the hyperpolarization. The responses to opioids were present in salines which contained nominally zero calcium and 6mM magnesium [in which e.p.s.p.s were abolished] suggesting that they were of postsynaptic origin. D-ala²-met⁵-enkephalin [DAME] and DADLE evoked similar responses from these neurones. Naloxone [100nM to 1uM] completely reversed the hyperpolarizations evoked by either normorphine or DAME.

A further intracellular study of rat locus coeruleus neurones confirmed that opioids evoked a hyperpolarization of the neuronal membrane potential [Williams, Egan and North, 1982]. These responses were not desensitized by prolonged application of opioid and were accompanied by a decreased membrane input resistance.

The hyperpolarizing responses evoked by opioids were apparently voltage dependent [Williams et al., 1982]; the amplitude was reported to be sharply reduced on depolarization of the membrane potential from resting levels. This effect was also seen in the presence of tetrodotoxin [TTX] and extracellular cobalt ions, suggesting that the opioid responses from these neurones were postsynaptic.

Williams and co-workers [1982] clearly showed that the reversal potential of the opioid responses was dependent upon the extracellular potassium ion concentration. These results suggested that the mechanism underlying this response was an increase in a potassium ion conductance. The resulting abolition of the hyperpolarization by either caesium ion injection into the neurone or extracellular perfusion of barium ions, corroborated this theory. Changes of the extracellular chloride ion concentration had no effect on this response implying that chloride ion movement did not contribute to this response. Their results led to the conclusion that opioids increased a potassium conductance in these neurones.

The opioid response was shown to be reproducible between neurones within the locus coeruleus. This feature allowed a determination of the receptor sub-type responsible for this response, [North and Williams, 1983a]. Application of opioids was by pressure ejection, the different doses being applied as multiples of a pressure pulse with a set duration. Using this method, a series of reproducible dose/response relationships were produced for opioid application to a neurone. However, the dose/response relationship was only specific for that particular neurone. Therefore, further

dose/response relationships were constructed for the same neurone with and without the presence of an opioid antagonist. They used a concentration of antagonist which ensured against any [measurable] direct interference with the resting membrane potential or membrane input resistance of the neurones [cf. for naloxone, Dingledine, Iversen and Breuker, 1978; Gruol, Barker and Smith, 1980]. From these experiments, North and Williams concluded that the Mu-subtype of opioid receptor played a major role in the hyperpolarizing response evoked by opioids from neurones in the rat locus coeruleus.

Action potentials produced by locus coeruleus neurones were also affected by the opioids. North and Williams [1983b] reported that opioids reduced the duration of the action potential. This effect resembled that produced by extracellular cobalt ions; a reduction in the duration of the repolarization phase of the action potential. These results suggested an interference with the calcium component of the action potential. To test this, action potentials were evoked in the presence of TTX. The TTX-insensitive action potentials were affected by normorphine or DADLE in 3 ways: the rate of rise and amplitude of the action potential were reduced and the amplitude of the after-hyperpolarization was also decreased. The presence of extracellular barium ions prevented all of those opioid effects. This was found even in low concentrations of barium ions which, by themselves, did not affect the action potential. The presence of caesium ions inside the cell also prevented the opioid effects on TTX-insensitive action potentials. It was concluded from these experiments that opioids produced an increase in a potassium ion conductance. This indirectly modulated calcium entry by shortening the duration of the action potential. North and Williams, [1983b]

reported that all of these effects could be reversed by naloxone and/or on washing out the opioid. It was further reported that prolonged opioid application did not desensitize these responses.

There is a problem with linking a drug effect in the brain with some aspect of the physiology of the animal. This problem is compounded by the complexity of interactions between the different brain regions. A simpler system, from which one can infer a possible physiological role, is the mammalian spinal cord where the peptides may have roles in the transfer of sensory information [Cervero and Iggo, 1980]. The effect of opioids on neuronal activity in the dorsal horn [where the sensory afferents terminate] of the mammalian spinal cord is the subject of the next section.

I.2.4] SPINAL NEURONES

Studies with met⁵-enkephalin were made on neurones in laminae II to V of the dorsal horn of the cat spinal cord [Duggan, Hall and Headley, 1976]. Neurones in laminae IV and V responded to the administration of noxious and/or innocuous stimulation of their peripheral receptive fields. During extracellular recording, the activity recorded from Laminae IV and V was increased in response to these peripheral stimuli. When met⁵-enkephalin was applied to laminae II or III [substantia gelatinosa] there was a reduction in the response to peripheral noxious stimulation from 12 of the 17 neurones tested, [Duggan *et al.*, 1976]. Of those 12 neurones: one showed a non-specific depression of activity on enkephalin application, 6 produced an inhibition in the absence of any response to noxious stimulation, and the remaining 5 showed a decrease in the

response to noxious stimulation and only a slight decrease in the response to innocuous stimulation. When met⁵-enkephalinamide was applied to laminae II and III it was reported as being consistently more selective in the depression of noxious stimulation-evoked activity than met⁵-enkephalin.

In a further study of the cat spinal cord, these workers reported that enkephalin application to laminae IV or V non-specifically depressed the peripheral stimulus-evoked activity [Duggan, Hall and Headley, 1977]. This became more evident the closer the site of opioid application was to the neurone from which the recordings were being made. Inhibition of peripherally evoked activity was also accompanied by a depression of spontaneous activity [9 of 19 tests]. Naloxone administered by an intravenous route only partially reversed these opioid-evoked depressions of activity. However, naloxone fully reversed these enkephalin evoked responses when it was applied directly to the dorsal horn by iontophoresis.

A later study on dorsal horn neurones of the cat spinal cord confirmed and extended those reports by Duggan et al., [1976,1977]. Enkephalin application into laminae II and III selectively inhibited the peripheral stimulus-evoked activity recorded from laminae IV and V, [Randic and Miletic, 1978]. Naloxone administration to lamina V did not inhibit the effects of enkephalin administration into laminae II and III. However, application of naloxone into laminae II and III antagonized the enkephalin-evoked depression of neuronal activity.

Both met⁵- and leu⁵-enkephalin evoked inhibitory responses from neurones in the dorsal horn of the cat spinal cord,

Zieglgansberger and Tulloch [1979]. In this study, the results for each of these peptides were almost identical and were therefore grouped together. Extracellular recordings were made from neurones in laminae IV, V and VI, where the majority of neurones tested with enkephalin responded with a depression of activity. Spontaneously active, synaptically-driven and L-glutamate-evoked activity was depressed by the enkephalins. Prior administration of naloxone produced a 60 to 100% reversal of enkephalin responses, [6 of 8 neurones]. These authors reported that naloxone alone did not affect the measurable parameters of the action potential. However, in some neurones naloxone did increase the background activity by between 10 and 30%.

In the same report Zieglgansberger and Tulloch [1979] also reported an intracellular study of neurones in lamina V of the cat spinal cord, in vivo. This study consisted of recordings from 20 neurones [9 from lamina V and 11 motoneurones]. No changes in the resting membrane potentials or the membrane input resistances of these neurones were found following the application of enkephalin. However, the effect of L-glutamate on the current/voltage relationship of the neurone was attenuated in the presence of enkephalin. Neurones were chosen for this study in which the current/voltage curves did not show any appreciable anomalous rectification at potentials of up to -100mV. These neurones also had input resistances from 2 to 10 Megaohms, [cf. similar studies by Murase and Randic [1983], who reported input resistances of 98 Megaohms].

The studies of opioid responses in the spinal cord which have

been described so far used the in vivo model with its inherent difficulties. However, the problems of access to the neurones have recently been overcome by using slice preparations of the spinal cord. The rat spinal cord slice preparation has allowed for a more detailed study of the opioid-evoked inhibitory responses of dorsal horn neurones. An intracellular study of neurones in lamina II of the dorsal horn was made by Yoshimura and North, [1983]. Normorphine and DADLE were applied separately to the slices by superfusion. Both of these compounds evoked a hyperpolarization which was accompanied by a decrease in input resistance, [13 of 26 neurones tested]. Yoshimura and North [1983] reported that this opioid response inverted at potentials more negative than the resting membrane potential of the neurone. The reversal potential was clearly shown to be dependent upon the extracellular potassium ion concentration and independent of the extracellular chloride ion concentration. Opioid responses were elicited from neurones bathed in a medium which abolished synaptic potentials. Naloxone [at a concentration of 3nM] substantially antagonized this hyperpolarizing response whilst not having any [measurable] direct effects on the membrane potential of the neurones. From these preliminary studies by Yoshimura and North [1983], it appears as though these opioid responses are similar to those reported for the neurones of the nucleus locus coeruleus [Pepper and Henderson, 1980; Williams et al., 1982] and result from an increase in a potassium conductance.

Ideally, if one wants to study the responses of the opioids in greater depth, one should be able to see the living neurones. However, the complexity of the mammalian CNS precludes the visualization of living neurones thus restricting studies of neuronal

electrophysiology; especially when one wishes to apply drug containing solutions discretely to known areas of a neurone. One method of overcoming this problem is by using a primary dissociated culture of the tissue. The most obvious advantage of cell culture is that the individual cells and processes can be seen; not just the cell bodies and thick dendrites sometimes visible at the edge of thick tissue explants [Wardell, 1966]. A further advantage is that the neurones are located on the top of a layer of non-neuronal cells and their uppermost surface is not covered by glia [Ransom, Neale, Henkart, Bullock and Nelson, 1977a]. This means the neurones can be "easily" impaled with microelectrodes, and there is no glial barrier between the neurone and recording or drug containing solutions [Ransom et al., 1977a]. The next section describes studies which were made on the opioid responses from neurones in such a primary culture.

I.2.5] SPINAL NEURONES IN DISSOCIATED CELL CULTURE

A variety of responses to enkephalin have been reported in spinal neurones from the mouse embryo, [Barker, Gruol, Huang, MacDonald and Smith, 1980a]. These neurones had been maintained in a primary dissociated cell culture, [Ransom et al., 1977a]. When the neurones were large enough to withstand intracellular recording [over 4 weeks in vitro] electrophysiological measurements were made. These studies employed either single electrode current clamp or double electrode voltage clamp techniques.

Although DRG neurones were present in these cultures, opioid responses from them will not be reported in this section. Those

results may be found in the following section.

The responses of the cultured spinal neurones to leu⁵-enkephalin were of four types, [Barker et al., 1980a, these workers only *formed* three groups. However, four categories allows a greater clarification of the responses].

I.2.5.1] "NEUROTRANSMITTER-LIKE" RESPONSES

The first type of responses consisted of those which were similar to direct/postsynaptic neurotransmitter actions. 10% of the neurones tested responded to *enkephalin* with a depolarization. These responses had a rapid onset and desensitized quickly on prolonged application. The onset was faster than that of similar depolarizations evoked from these neurones by L-glutamate. Extrapolated reversal potentials for this response varied, from -4mV, +10mV to even +20mV, [Barker et al., 1978a; Barker et al., 1980a; Barker, Gruol, Huang, MacDonald and Smith, 1980b]. These opioid-evoked depolarizations were capable of generating action potentials.

Further "neurotransmitter-like" responses were reported as hyperpolarizations. These responses had reversal potentials in the range -40 to -60mV and were found in a third of those neurones tested by Barker and co-workers, [1978a]. These hyperpolarizations were associated with small increases in membrane conductance, [approximately 20%]. A later study by this group showed fewer neurones giving this type of response to enkephalin, [only 22 of 128 neurones tested by Barker et al., 1980a]. These enkephalin effects

were similar to the responses evoked from these neurones by GABA [Barker and Ransom, 1978]. Raising the intracellular chloride ion concentration affected both the GABA and the enkephalin-evoked hyperpolarizing responses, [Barker et al., 1980a]. However, the hyperpolarizing responses to enkephalin were attenuated by naloxone whereas GABA responses were not. [but cf. Dingledine et al., 1978; Gruol et al., 1980]. These hyperpolarizing responses evoked by enkephalin did not show appreciable desensitization on repeated or prolonged application.

Report was made of a depolarization which was associated with little if any change in membrane conductance, [Barker, Smith and Neale, 1978b]. However, this possible enkephalin response was not studied in detail, [only 4 of 37 neurones tested responded in this way]. Barker et al., [1978b] also reported that these depolarizations were accompanied by an increase in the excitability of the neurone. This increase in excitability was attenuated by naloxone [2 of 2 tests].

I.2.5.2] ABRUPT DEPOLARIZING EVENTS

The second variety of action described for leu⁵-enkephalin on these neurones consisted of abrupt depolarizations, [cf. the prolonged depolarizations of hippocampal pyramidal cells in culture, Gahwiler, 1980]. These were evoked from 10% of the neurones tested, [Barker et al., 1978a]. The depolarizations usually occurred in steps of circa 10mV. Changes in the current flowing through the cell membrane during these events were monitored using voltage clamp techniques, [Barker et al., 1978b]. Total changes in current flow

were up to 10nA. These abrupt depolarizations were not attenuated by naloxone, [3 neurones]. However, these events were usually preceded by one of the three "standard neurotransmitter-like" actions evoked by the enkephalins [vide supra].

I.2.5.3] AFFECTS ON ACTION POTENTIAL GENERATION

This was reported as an alteration of the threshold potential for action potential generation. Only 29 of 128 neurones tested with leu⁵-enkephalin gave this type of response [Barker et al., 1980a]. 20 of those 29 neurones responded with an elevated threshold for action potential generation. Occasionally the enkephalins totally suppressed the ability of a neurone to generate action potentials. There was little, if any, measurable change in the current/voltage relationship for the neurone during this type of enkephalin response.

A further 5 of the 29 responsive neurones did so with an elevation of the threshold potential for action potential generation. However, these effects were accompanied by an increase in the conductance of the neurone. Barker et al., [1980a] reported that moving the drug application electrode away from the impaled neurone selected for the elevation of action potential threshold effect over the increase in conductance. In the 4 remaining neurones the threshold potential was depressed by enkephalin. No associated membrane effects were reported with the latter effect. All of these events were insensitive to naloxone and were not desensitized by prolonged application of enkephalin.

I.2.5.4] MODULATION OF AMINO ACID RESPONSES

These consisted of a postsynaptic modulation of amino acid responses. The amino acids in question were L-glutamate, GABA and glycine. Barker, Neale, Smith and MacDonald, [1978c] reported the depression of L-glutamate responses by enkephalin, found in 28 of 32 neurones. This depression was independent of other effects relating to the neuronal membrane properties and was reported to be partially reversible by naloxone [6 of 10 tests]. The maximum restoration by naloxone was to within 80% of the normal L-glutamate response. The depression of L-glutamate responses by enkephalin was associated with a "slowing down" of the time course of the amino acid responses. Barker et al., [1978c] described a study of leu⁵-enkephalin on the dose/response relationship for the L-glutamate response. They concluded that the action of enkephalin was non-competitive. However, these workers also reported that enkephalin did not alter either the receptor affinity for L-glutamate or, the number of L-glutamate molecules involved in the production of the unitary conductance.

The modulatory effects of enkephalins on GABA or glycine responses have not been studied in such detail. Leu⁵-enkephalin predominantly caused a depression of the GABA response, [9 of 15 neurones]. However, Barker et al., [1978b] described a slight enhancement of the GABA response when low doses of enkephalin were used, [3 of 9 neurones]. The glycine response was affected in a similar fashion by leu⁵-enkephalin; e.g., low doses of enkephalin enhanced the glycine response, [5 of 18 neurones] and at higher doses of enkephalin, the glycine response was depressed.

I.2.6] DORSAL ROOT GANGLION NEURONES IN DISSOCIATED CELL CULTURE

Primary dissociated cell cultures were produced from embryonic mouse dorsal root ganglia and spinal cords. Intracellular recordings were made from cultured sensory neurones, [Werz and MacDonald, 1982a]. Action potentials were evoked from these neurones under normal conditions and in the presence of enkephalin. In the presence of leu⁵-enkephalin at concentrations of between 20nM and 5uM, the duration of action potentials from these sensory neurones was reduced. The effects of leu⁵-enkephalin [1uM] were reversibly antagonized by naloxone [1uM]. Prolonged opioid application apparently did not desensitize this response. The proportion of responsive neurones varied greatly between preparations.

In a more complete study, the selectivity of neurones was tested with low concentrations of morphiceptin and/or leu⁵-enkephalin. In these experiments low concentrations of naloxone were shown to reverse some of the opioid responses but not others, [Werz and MacDonald, 1982c;1983a]. The inability of naloxone to reverse the action of leu⁵-enkephalin on some neurones corresponded with those neurones which did not respond to morphiceptin. This led to the conclusion that more than one type of opioid receptor mediated the response evoked from these neurones. Tested alongside the opioids were noradrenaline and cadmium ions. Both the noradrenaline and the cadmium ions also reduced the action potential duration. These effects appeared similar to those produced by the opioids. In an attempt to elucidate the underlying mechanism of action Werz and MacDonald [1983b] studied these responses before and after intracellular loading of the neurones with caesium ions. Opioid

application to neurones loaded with caesium ions did not affect the action potential duration. However, the noradrenaline response was enhanced in the caesium loaded neurones. Cadmium ions greatly reduced the action potential duration in both normal and caesium loaded sensory neurones.

The responses to noradrenaline reported by Werz and MacDonald [1983b] were similar to results from studies on chick sensory neurones [Dunlap and Fischbach, 1981]. However, the opioid responses from mammalian sensory neurones differed in several ways from those of chick sensory neurones, [Mudge et al., 1979; Dunlap and Fischbach, 1981]. After opioid application to mammalian sensory neurones the action potential after-hyperpolarization was increased, [Werz and MacDonald, 1983b], whereas after either noradrenaline or cadmium ions it was decreased. These results suggested that the opioid response from mouse DRG neurones in culture was mediated by an increase in a potassium conductance, which was possibly voltage and/or calcium dependent.

Further studies on mammalian sensory neurones in dissociated culture have shown the presence of a noradrenaline-like reduction in action potential duration produced by an opioid. This effect was reported for dynorphin, a "selective" Kappa-opioid receptor agonist [Werz and MacDonald, 1984]. These opioid responses were separate from the Mu and Delta mediated effects on action potential duration, and were reported as being found occasionally in the same neurone. Thus it appears that there are 3 types of opioid receptor present on different sub-populations of DRG neurone in culture. The Mu and Delta apparently acting to increase a voltage and/or calcium

sensitive potassium conductance, whereas the Kappa appears to reduce a calcium conductance. A similar response to that mediated by the Kappa receptor was described for the opioids effects on chick sensory neurones in culture [Mudge et al., 1979].

Mudge et al., [1979], Dunlap and Fischbach, [1981] and Werz and MacDonald, [1982a,b,c; 1983a,b; 1984] have described two different mechanisms through which the opioids affect the action potential duration in dorsal root ganglion neurones. In contrast, Williams and Zieglgansberger, [1981] reported that opioids did not affect the action potential duration of neurones in the adult rat dorsal root ganglion, in vivo. This may mean these opioid effects were artefacts of tissue culture. However, more realistic possibilities are that as the neurones mature they lose the ability to produce this type of response [by a reduced access of the drug to the neurone], or that the type of opioid receptor activating the response changes with age [cf. Wohltmann, Roth and Coscia, 1982].

The multiple actions which were evoked from the cultured neurones by opioids have not been fully studied. These actions, along with the possible modulation of amino acid responses, give the opioids many possible mechanisms for interfering with inter-neuronal communication. By using neurones in a spinal cord culture [cf. Barker et al., 1978a] these responses could be further studied in detail.

I.3] Phe-Met-Arg-Phe-NH₂

I.3.1] ISOLATION AND CHARACTERIZATION

A "molluscan cardioexcitatory" neuropeptide was isolated by Price and Greenberg, [1977] in extracts of ganglia from the clam, Macrocallista nimbosa. Characterization and amino-acid sequencing of the extract gave an amidated tetrapeptide, Phe-Met-Arg-Phe-NH₂ [FMRFamide using single letter abbreviations; IUPAC-IUB, 1972].

Dockray, Vaillant and Williams, [1981] used an antibody which recognized the Arg-Phe-NH₂ [C-terminus] of FMRFamide. They reported that FMRFamide-like immunoreactivity was located only in "nerve fibres" in the rat CNS. This immunoreactivity was distinct from that of either met⁵-enkephalin or cholecystokinin. Weber, Evans, Samuelsson and Barchas, [1981] also used an antibody which was reported to recognize Arg-Phe-NH₂ but [in contrast to Dockray et al., 1981] they found a "whole" neuronal system in the rat CNS. Cell bodies were reported in cortical, limbic and hypothalamic areas. Fibres were described as widespread throughout the brain, spinal cord and pituitary. This system was distinct from those found for either met⁵-enkephalin or the heptapeptide [YGGFMRF]. The results of these studies suggested the existence of a FMRFamide-like peptide in the mammalian CNS.

I.4] NEURONAL PHARMACOLOGY OF FMRFamide

The results of studies in invertebrate systems are included in the following section as FMRFamide was initially isolated and localized in invertebrates. In addition to this, up until now, intracellular electrophysiological studies had been performed solely in invertebrates.

I.4.1] INVERTEBRATE

Early studies into the neuronal pharmacology of FMRFamide were made in the snail, Helix aspersa.

Cottrell [1980] made a study of FMRFamide responses from an identified neurone in the cerebral ganglion of the snail, Helix aspersa. Intracellular recordings were made from the C1 neurone and FMRFamide was applied to this neurone by iontophoresis. At resting membrane potential the response of the C1 neurone to FMRFamide was a hyperpolarization. It was shown, by using voltage clamp, that the hyperpolarization was accompanied by an outward current moving across the cell membrane. This response was blocked by tetraethylammonium ions [TEA] and enhanced by a reducing the extracellular potassium concentration [Cottrell, 1982b]. The enhancement caused by a reduction of the extracellular potassium concentration was due to a change in the equilibrium potential of the response. Changes in the extracellular chloride concentration had no effect on the size of the hyperpolarization. When these results were taken together they suggested that the mechanism underlying this response was an increase in a potassium conductance.

At more positive membrane potentials the response became a depolarization which was accompanied by an inward current [Cottrell, 1980]. This response was less sensitive to extracellular TEA or to reductions in the extracellular sodium or chloride ion concentration, [Cottrell, 1982b]. However, the addition of 1.5mM cobalt chloride to the extracellular medium abolished this response. In addition, it was reported that replacement of most of the external calcium ions with barium ions abolished the response. These results suggested that a calcium activated conductance was being affected. The ability of barium ions to abolish the response implied that potassium was involved. These results, added to the observed decrease in the overall current flowing through the membrane during this response, [Cottrell, 1982b] lead to the conclusion that the depolarizing response was possibly mediated by the reduction of a calcium dependent potassium ion conductance. Apart from the responses mediated by alterations in potassium conductance, a further report has described actions which appeared to be mediated by an increase in a sodium ion conductance [Cottrell, Davies and Green, 1984]. There were two responses with a sodium ion component. The response from the F2 neurone was a combination of sodium and potassium ion conductance increases. These responses appeared as depolarizations, the reversal potential being between -50 and -25mV in different neurones. A further complication with these responses was the desensitization after repeated application. FMRFamide application to the E13 neurone also evoked a depolarization. However, these responses had reversal potentials which were more positive than +20mV. Experiments with extracellular cobalt [1mM], sodium-free medium and TEA suggested that sodium ions played the major role in these responses with apparently no potassium or calcium component.

I.4.2] MAMMALIAN

Electrophysiological evidence for the involvement of a FMRFamide-like compound in mammalian neurotransmission came from an electrophysiological study of the rat brainstem, in vivo [Gayton, 1982]. In this study, a comparison was made between the effects of FMRFamide, FMRF and the opioid heptapeptide [YGGFMRF using single letter abbreviations; IUPAC-IUB, 1972] on extracellularly recorded neuronal activity. FMRFamide predominantly increased the rate of firing of neurones in the brainstem. The excitatory response was of a rapid onset and recovery and was not attenuated by naloxone. Only one neurone in this study responded to FMRFamide with a decrease in spontaneous activity. In contrast to this, the non-amidated tetrapeptide [FMRF] was reported to evoke little or no change in the spontaneous activity. YGGFMRF produced either increases or decreases of the activity recorded from different neuronal units. The majority of the responses evoked by the opioid heptapeptide were depressions of activity with a slow onset and recovery. All of these heptapeptide effects were attenuated by naloxone.

In a similar study, Gayton [personal communication] reported that a lower percentage of neuronal units responded to FMRFamide in the midbrain than in the brainstem. The majority of responses from midbrain neurones were excitatory. As in the brainstem [Gayton, 1982], no biphasic responses to FMRFamide were reported from the midbrain. The results of these studies suggest that a FMRFamide-like compound may be involved in neurotransmission in the mammalian CNS. The FMRFamide was apparently not acting via an opioid system in the rat brainstem.

Three features led to the use of FMRFamide in this thesis. The first involved the obvious similarity between it and the opioid heptapeptide [YGGFMRF] from bovine adrenal glands. This suggested the possibility of FMRFamide sharing some responses with the opioids. The finding of fibres in the spinal cord which contained FMRFamide-like immunoreactivity suggested the possibility of a modulatory role for a FMRFamide-like peptide. The third feature came from the study by Gayton [1982], where FMRFamide was shown to alter neuronal activity in the rat brainstem.

I.5] NEUROTENSIN

I.5.1] ISOLATION AND CHARACTERIZATION

The discovery and isolation of a tridecapeptide peptide from extracts of bovine hypothalamus was reported by Carraway and Leeman, [1973]. When this peptide was injected intravenously into rats it produced hypotension, vasodilation and cyanosis. Location of this peptide in nervous tissue and the resulting hypotensive action after injection into rats led to it being named neurotensin. Bovine neurotensin was characterized as a tridecapeptide with the amino acid sequence pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH, [Carraway and Leeman, 1975]. This peptide also showed activity in a variety of in vitro preparations. It caused contractions in preparations of guinea-pig ileum and rat uterus and relaxed in vitro preparations of the rat duodenum.

I.6] PHARMACOLOGY OF NEUROTENSIN IN THE MAMMALIAN CNS

The majority of the studies with neurotensin have used extracellular recording from neurones with the associated problems in interpretation [cf. the enkephalins, II] the hippocampus]. These studies have been split into those performed in vivo and those in vitro.

I.6.1] in vivo STUDIES

I.6.1.1] BRAIN

It was originally claimed that neurotensin evoked no responses from neurones in the locus coeruleus of the rat [Guyenet and Aghajanian, 1977]. This study used multibarrelled electrodes to apply the neurotensin and studied only a small number of cells. A further study by Young, Uhl and Kuhar [1978] found that the majority of rat locus coeruleus neurones did respond to neurotensin. In the latter study, neurotensin produced depressions of the spontaneous and/or L-glutamate-evoked neuronal activity. These neurotensin responses were of rapid onset and quickly recovered on termination of peptide application. Repeated applications of neurotensin apparently led to the desensitization of these inhibitory responses.

Excitations of neuronal activity were reported [Phillis and Kirkpatrick, 1980] following the application of neurotensin to the rat cerebral cortex. These responses were recorded extracellularly and appeared as weak increases in neuronal activity which had a slow onset. These increases in activity occurred in 25% of those neurones

classified as corticospinal and 25% of the unidentified neurones. Neurotensin did not depress neuronal activity in that study. In an attempt to study interactions between neurotensin and an amino acid, the peptide was applied in conjunction with repetitive pulses of L-glutamate. The only change to the L-glutamate response was an occasional increase in size.

Both depressions and excitations of neuronal activity were reported by McCarthy, Walker, Yajima, Kitagawa and Woodruff, [1979] in an extracellular study of the nucleus accumbens and cerebellum of the rat brain, in vivo. The application of neurotensin, by iontophoresis, evoked a potent depression of activity from neurones in the nucleus accumbens. This effect had a rapid onset and recovery. However, only a few cerebellar neurones responded to neurotensin. At higher ejection currents the peptide evoked a slow onset and weak depression of activity from those neurones. A further study of rat cerebellar Purkinje cells, in vivo, by Marwaha, Hoffer and Freedman, [1980] showed that neurotensin evoked a reduction in neuronal activity. These workers reported that the response evoked by pressure ejected neurotensin was greater than that produced by iontophoreted neurotensin.

The inhibitory responses evoked from cerebellar Purkinje cells by neurotensin were abolished by the presence of magnesium ions or haloperidol in the extracellular medium, [Marwaha et al., 1980]. Pretreatment of the rat brain with 6-hydroxydopamine also abolished these inhibitory responses. In the presence of either haloperidol or high extracellular magnesium and in those brains which had been pretreated with 6-hydroxydopamine; the application of neurotensin

evoked slow excitatory responses from the cerebellar Purkinje cells. The results from this study suggested that the depressant activity had a presynaptic origin and that neurotensin acted to release a monoamine which then caused the inhibition of neuronal activity. The excitatory effects were suggested as being the postsynaptic response of these cerebellar Purkinje cells to neurotensin.

I.6.1.2] SPINAL CORD

When neurotensin was applied to neurones in laminae I-III of the cat spinal cord it evoked an increase in neuronal activity, [Miletic and Randic, 1979]. These responses were weak excitations of both the spontaneous and/or drug evoked activity. Application of peptide even initiated the generation of action potentials from some quiescent neurones. Both the onset of the response and the recovery, after termination of peptide application, were slow.

A study was made of the effects of neurotensin on neurones which also respond to various types of peripheral stimulation. It was found that the neurones affected by neurotensin could also respond to either noxious or innocuous peripheral stimulation. Miletic and Randic [1979] concluded from this that the peptide effects were apparently not specific to neurones involved in the transmission of noxious stimuli. Neurotensin was reported as having no effects in laminae IV-VII.

In a later study of dorsal horn neurones from the cat spinal cord, the majority of neurotensin responses were reported as depressions of neuronal activity, [Henry, 1982]. These inhibitory

responses were of rapid onset and recovery. Only one neurone tested in this study responded with an increase in activity. The low number of excitatory effects caused by the peptide in this study was possibly a reflection of the low overall sample size, only 16 responsive neuronal units. However, the excitatory response was strong, unlike those described by Miletic and Randic [1979]; increases in firing rate were up to 200% that of resting levels. In Henry's [1982] study, the neurotensin effects were found in Laminae I, III and IV but not in Lamina II. This again contrasts with Miletic and Randic [1979], who found neurotensin responses in laminae I-III.

A study of neurotensin responses from neurones in the dorsal horn of the in vivo rat spinal cord was performed by Stanzione and Zieglgansberger, [1983]. Neurotensin was applied by either iontophoresis or pressure ejection. Extracellular recordings were made from 20 neurones. All of these neurones were excited by neurotensin application. These responses were dose-dependent, showing only a slight amount of desensitization after repeated application of peptide. The excitations evoked by L-glutamate were additive to those evoked by neurotensin.

Twenty-five neurones were tested in an intracellular study of the dorsal horn of the rat spinal cord [Stanzione and Zieglgansberger, 1983]. Neurotensin was either pressure ejected or iontophoresed onto these neurones. Application of this peptide evoked depolarizations of the membrane potential in 13 of the 25 neurones. No hyperpolarizing responses were reported for these neurones. An increase in the input resistance accompanied the

depolarizing response. This change in resistance was described as following the onset of the depolarization by a "few seconds".

I.6.2] in vitro STUDIES

Neurotensin-evoked excitations of neuronal activity from the bed nucleus of the guinea-pig stria terminalis, [Sawada, Takada and Yamamoto, [1980]]. The peptide was applied to the slices of brain by microperfusion. Microperfusion enabled the workers to apply known concentrations of peptide to the neurones whilst making extracellular recordings. The activity of approximately 66% of the neuronal units tested was increased by neurotensin. An increase in recorded activity was evoked from these neurones by neurotensin even in the presence of low calcium/high magnesium concentrations in the extracellular medium. No inhibitory responses were described for the peptide in this report. It was concluded that the excitatory response was of a postsynaptic origin.

Neurotensin-like immunoreactivity has been found in the dorsal horn of the mammalian spinal cord [Seybold and Elde, 1980; Polak and Bloom, 1982]. In addition, this section has described the reports from a number of workers who have shown that neurotensin can evoke responses from spinal neurones [Miletic and Randic, 1979; Henry, 1982; Stanzione and Zieglgansberger, 1983]. However, little has yet been discovered concerning the mechanism[s] of action of this peptide. Neurotensin has also affected neurones which were capable of responding to peripheral stimulation, [Miletic and Randic, 1979], thus suggesting it has a role as a modulator of sensory input. Therefore, this peptide became a candidate for testing on the

cultured mouse spinal neurones.

I.7] GLYCYL L-GLUTAMINE

I.7.1] ISOLATION AND CHARACTERIZATION

The dipeptide glycyl L-glutamine was discovered and characterized by Parish, Smyth, Normanton and Wolstencroft, [1983]. These workers extracted this dipeptide from porcine pituitary glands. This dipeptide was later found in extracts of the sheep brainstem region which included the medulla, pons and midbrain. It was suggested that glycyl L-glutamine was derived from the C-terminal of the opioid peptide, B-endorphin.

I.8] NEURONAL PHARMACOLOGY OF GLYCYL L-GLUTAMINE

The effects of glycyl L-glutamine were tested on neuronal activity of the rat brainstem, [Parish et al., 1983]. In an extracellular study these workers found that the dipeptide inhibited the activity of 41 out of the 109 neurones tested. These 41 neurones were situated in the brainstem reticular formation and most of the responses were of a slow onset and recovery. However, circa 10% appeared more rapid than the rest, resembling the responses evoked from this area by GABA. The inhibitions of activity evoked by glycyl L-glutamine were not attenuated by the simultaneous application of either strychnine or naloxone. This suggested that glycine was not responsible for the inhibitory effect of the dipeptide. These actions of glycyl L-glutamine may have been caused by either, a

direct interaction with a membrane bound receptor to evoke a response, or the interaction with a receptor to prevent a response. The latter possibility came from the structural relationship between glycyl L-glutamine and L-glutamate antagonists; gamma-D-glutamyl glycine and glutamyl diethyl ester [Davies and Watkins, 1981; Watkins and Evans, 1981]. As spinal neurones in culture are capable of responding to L-glutamate [Ransom, Bullock and Nelson, 1977b; MacDonald and Wojtowicz, 1982] and respond to peptides [Barker et al. 1978a; Nowak and MacDonald, 1982], this preparation would appear ideal for studying the possible direct and indirect roles of this peptide on spinal neurones.

I.9] CONCLUSIONS AND PROJECT

From the data presented in this chapter, it can be seen that the enkephalins [met⁵- and leu⁵-], FMRFamide, neurotensin and glycyl L-glutamine affect the activity of neurones in the mammalian CNS. However, little is known, as yet, about the underlying mechanisms through which these peptides affect the neurone. A major barrier to this research has been the complexity of the intact CNS. One of the main problems, that of access to the neurones, has been reduced by using slice preparations of CNS tissue. However, both the inability to visualize the area of drug application and, the relationship between the impaled neurone and nearby cells, can still be a problem. One way to overcome such a problem is to dissociate the cells and maintain them, as a monolayer, in tissue culture. The use of primary dissociated cell cultures allows the neurones to be visualized whilst still viable. The ability to visualize living cells has the

advantage of allowing the discrete application of drug solutions to the neurone under study [Ransom et al., 1977b]. This has already been shown in the study of opioid peptides and substance P by Barker and co-workers [1980a]. Therefore, the primary dissociated cell culture appears to be suitable for studying the actions of peptides on spinal neurones. The proposed study was of met⁵- and leu⁵-enkephalins, FMRFamide, neurotensin and glycyl L-glutamine. The chosen tissue was the spinal cord from the mouse embryo. The reason for this choice was the availability of previously published data with which comparisons of the general morphology, electrophysiology and chemosensitivity to amino acids [gamma-amino butyric acid, glycine and L-glutamate] could be made.

CHAPTER II

METHODS I

PREPARATION AND MAINTENANCE OF TISSUES FROM THE
MOUSE EMBRYO IN A PRIMARY DISSOCIATED
CELL CULTURE

II.1] ASEPTIC TECHNIQUE

One of the most important features of these cultures was that they were maintained in media which did not contain any antibiotic or fungicidal agent. This meant there was a need for asepsis throughout the preparation and maintenance of the cultures. Stringent adherence to the following procedures was crucial for the successful preparation of these cultures. Without the following precautions the number of viable cells produced by the dissection was greatly reduced with an increased risk of the resulting cultures being contaminated.

A] All solutions used in conjunction with the cultures were "filter sterilized" by passage through a 0.2um filter unit [Millipore].

B] All instruments were sterilised in an autoclave. During the dissection, when the instruments were not in use, they were kept in a container of 70% alcohol.

C] Wherever possible, the bench-space chosen for the dissection were relatively dust and draught free.

D] Prior to use, bench surfaces and non-sterilized equipment [microscope etc.] were rinsed or swabbed with 70% alcohol.

E] People were actively dissuaded from entering or passing the area whilst dissections were in progress.

F] Breathing directly over the preparation was avoided during the dissection.

G] Any 70% alcohol which came into contact with the tissue caused cell death. Therefore, utmost care was taken to prevent this contact occurring. The use of 70% alcohol was stringently controlled.

H] Gloves were worn throughout the dissection. These gloves were changed after removing the gravid uterus from the mouse, prior to working inside the laminar flow cabinet [Gelaire class 100, Gelman instruments] and at any other time when contamination was thought to have occurred.

II.2] DISSECTION

MEDIUM USED DURING THE DISSECTION [DISSECTING MEDIUM]

This was prepared by the addition of 50ml of Hanks balanced salt solution without calcium or magnesium [x10 concentrate] to 50ml of a 12% glucose- 40% sucrose solution [made in distilled water]. A 28ml aliquot of Hepes [Sigma] buffer solution [325mM] which was at

pH 7.3- 7.4 was then added to the salt solution. This mixture was diluted to 975ml with distilled water and the pH then re-adjusted to between 7.3 and 7.4. The osmolarity of that solution was then altered to between 320 and 340mOsm by the addition of either distilled water or solid sodium chloride. The volume of that solution was then increased to 1 litre. After filtration through a 0.2um filter unit [SYBRON, Nalge] the solution was ready for use as a dissecting medium.

II.2.1] EXTRACTION OF THE GRAVID UTERUS.

The mouse was asphyxiated by exposure to carbon dioxide and the neck broken by cervical dislocation. It was rinsed in 70% alcohol, which reduced the risk of contamination, and then transferred on to the dissection bench, where it was placed on a paper tissue. This tissue had been previously soaked with 70% alcohol and was contained in a stainless steel tray.

Using a pair of large, blunt-tip scissors an incision was made through the skin of the abdomen. Care was taken not to cut through the peritoneum at this time. The incision was enlarged by tearing and 70% alcohol poured over the area to remove any loose hairs or dust. The abdominal contents of the mouse were now distinguishable. Intestines appeared yellow and lay below the red of the liver. The gravid uterus was also red and lay interspersed with the intestine in the lower half of the abdomen.

FIGURE II.1

A] UTERINE HORNS IN SITU

The arrow indicates the gravid uterus after it has been teased out of the abdomen.

Scale bar = 1cm.

B] The uterus laid on tissue after excision from the mother. The single arrow indicates a placenta.

Scale bar represents 1cm.

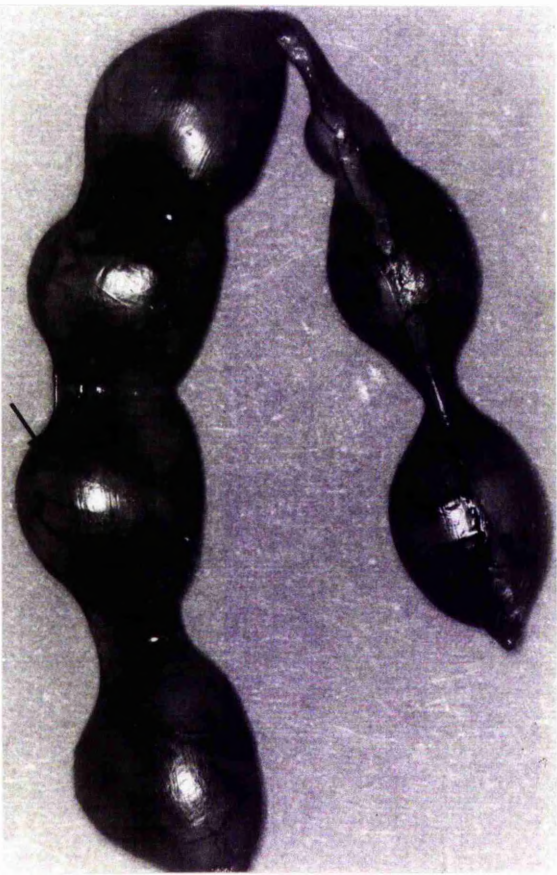
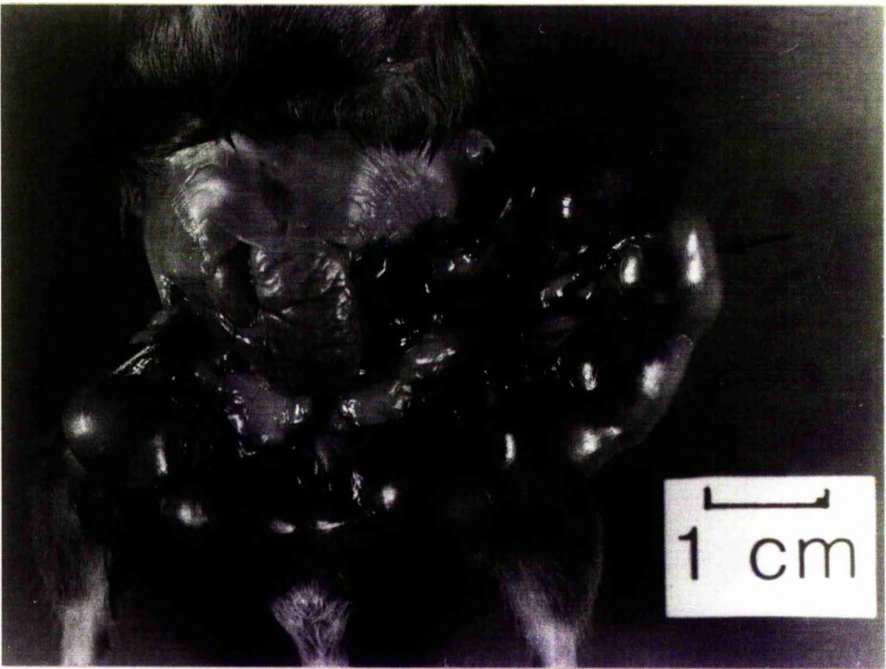
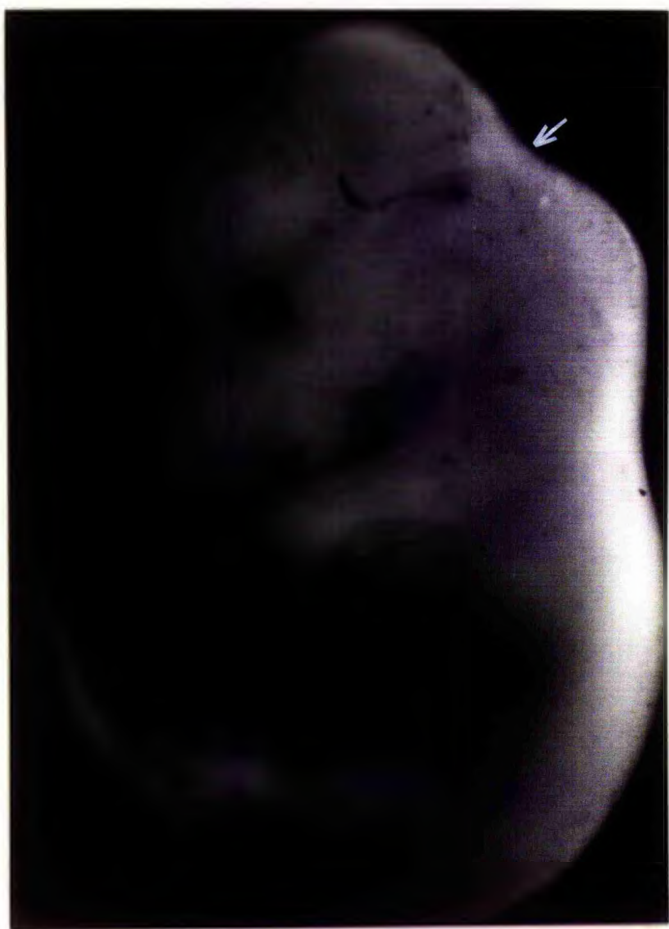


FIGURE II.2.

AN EMBRYO

This photomicrograph shows a 13.5 day old embryo. It was positioned ready for the removal of the spinal cord. The arrow indicates the point of the first incision.

Scale bar indicates 1mm.



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FIGURE II.3

A] AN ISOLATED BRAIN

The brain as it appeared after removal from the embryo. Arrow indicates the point of severance from the spinal cord.

Scale bar = 2mm

B] AN ISOLATED SPINAL CORD, VENTRAL VIEW

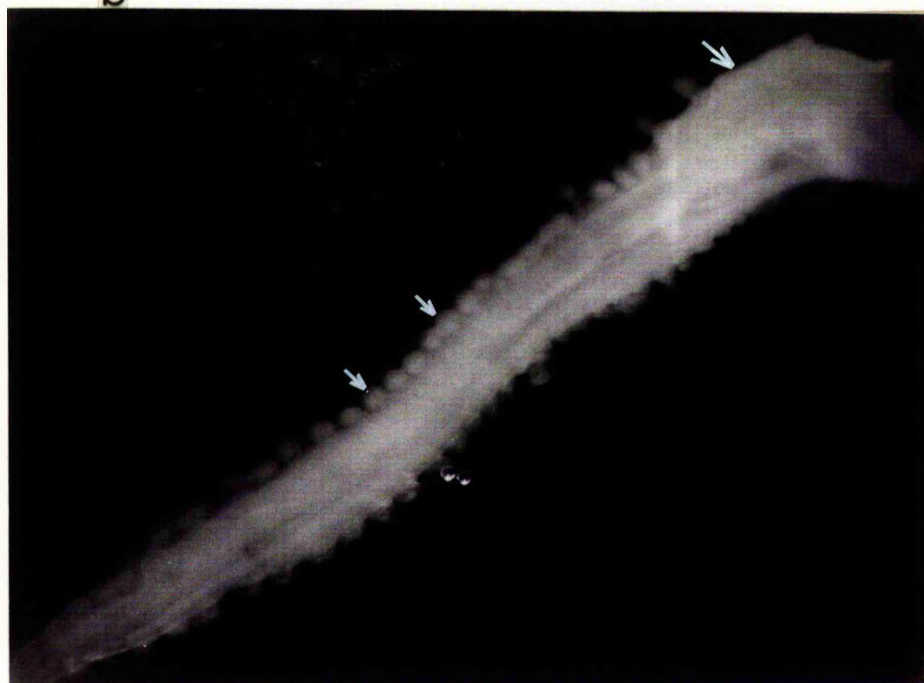
The spinal cord and attached ganglia [small arrows]. The large arrow marks position of the brainstem.

Scale bar = 1mm.

a



b



Part of the peritoneum overlying the uterus was lifted using blunt forceps. An incision was made through the peritoneum towards the uterus. Now the viscera could be clearly seen and care was taken to avoid their contact with the scissors. The incision was extended laterally from the midline in both directions. This exposed as much of the abdomen as possible. The abdominal contents were flooded with 70% alcohol and the gravid uterus teased out gently and positioned as in figure II.1a.

An incision was made between the left-hand uterine horn and its ovary. This was extended towards the right-hand ovary parallel to the uterus; through the mesentery and vagina. The connection between the uterus and the right hand ovary was severed. Once free of the mother, the gravid uterus was rinsed with 70% alcohol and placed on a clean piece of alcohol-soaked tissue [fig II.1b].

II.2.2] REMOVAL OF THE EMBRYO

Using blunt forceps and scissors an incision was made above the first embryo, on the placental side of the uterus, figure II.1b. An extension of this, along the uterus, exposed the chorionic vesicles. The chorionic vesicles were teased out of the uterus with great care. Vesicles which had ruptured were discarded; those embryos were probably contaminated or at least damaged having come into contact with the alcohol. A portion of the placenta usually remained attached to the chorionic vesicle at this point. This placenta and a pinch of chorionic vesicle were held with the blunt forceps and then lifted above the tray. The chorionic vesicle was then ruptured with the tips of Dumont No.5 forceps. The embryo was gently squeezed out and teased free until attached only by its umbilical cord. Using

forceps, the embryo was gently pulled away from the placenta. This action broke the umbilical cord which freed the embryo. Embryos were collected in a 35mm culture dish [Sterlin] containing dissecting medium. The culture dish and embryos were then transferred to the stage of a dissecting microscope.

The following dissections took place at a magnification of x25.

II.2.3] SPINAL CORD DISSECTION

Any damage to the spinal cord during the earlier stages of the dissection prolonged subsequent stages and therefore, increased the risk of contamination and cell death.

The embryo was positioned to face left, [fig II.2]. The tips of Dumont No.5 forceps were then pushed through the eye and lower limb of the embryo to secure it. If the brain was required, the head was secured using the lower jaw instead of the eye. Iridectomy scissors were used to make an incision at the rostral end of the spinal cord. This was just below the characteristic bulge of the mesencephalon and the metencephalon which was easily visible through the translucent tissue of the embryo. A caudal extension of the incision was made slightly ventral to the spinal cord.

The spinal cord was teased free of adhering tissue with the iridectomy scissors until it appeared as in fig II.3b. It was then transferred to a culture dish containing fresh dissecting medium and put into the laminar flow cabinet to await trypsinization.

II.2.4] CO-CULTURING TISSUE WITH THE SPINAL CORD CELLS

The other tissues, which had also been dissected from the embryos, were treated separately throughout the trypsinization and trituration stages. They were mixed with spinal cord cells just before plating out into the culture dishes. This was for two reasons: firstly, so a control culture of only spinal cord tissue could be set up and secondly, so that the proportions of each type of tissue in the culture would be known.

All cell suspensions were agitated prior to mixing and plating to ensure that the cultures were as homogeneous as possible.

II.2.5] BRAIN DISSECTION

The Dumont No.5 forceps were used to immobilize the head. Both tips pierced the head below the level of the brain. The first incision made for the spinal cord dissection was extended ventrally. Scissors were then used to tease the layers of embryonic skin, skull and meninges away from the brain. Once totally exposed, the brain could be removed by slowly teasing it away from the remaining tissue. Any areas of adhesion were severed with the scissors.

The brains were collected together in a fresh culture dish containing dissecting medium and transferred to the laminar flow cabinet. At this time the brains appeared as in fig II.3a.

II.2.6] HEART DISSECTION

The embryo was positioned with its dorsal surface uppermost and the thorax was opened using the iridectomy scissors. The embryo heart was visible along with the lungs, oesophagus and thymus gland. The heart was taken and used in the culture only if it was still beating on removal from the embryo. Tissue was cleared from around the heart. Incisions were made through the major blood vessels and then the heart was lifted out with the Dumont forceps. The hearts were collected in a 35mm diameter culture dish containing fresh dissecting medium.

II.2.7] ILEUM DISSECTION

The embryo was positioned as for the removal of the heart and the remaining abdominal skin teased away with the Dumont forceps. The gastrointestinal tract was uncoiled and an incision made between the ileum and the stomach. At the distal end an incision was made between the ileum and the colon [which was slightly larger in diameter than the ileum]. The tissue was then collected in a 35mm diameter culture dish containing fresh dissecting medium.

II.2.8] HIND LIMB DISSECTION

The embryo was positioned facing left. An incision was made through the top of the hind limb at its point of entry into the torso/trunk. The hind limb was separated from the torso, whereupon the hind paw was severed and discarded. The tissue surrounding the embryonic bone was removed and collected in a 35mm diameter culture dish containing fresh dissecting medium.

II.3] PREPARATION OF THE CULTURE

MEDIA USED TO MAINTAIN THE CELLS IN CULTURE

The modified Eagles medium containing Earles salts [MEM] was obtained from GIBCO. This required supplements of glucose, sodium bicarbonate and L-glutamine to give final concentrations of 33, 44 and 2mM respectively. A bottle containing 500ml of MEM [GIBCO] required 2.5gms of glucose, 1.425gms of sodium bicarbonate and 0.146gms of L-glutamine [or 5ml of the stock [200mM] L-glutamine (GIBCO)]. The final osmolarity of this solution was adjusted to between 320 and 340mOsm by the addition of either solid sodium chloride or distilled water. The final corrected MEM was "sterilized" by passage through a 0.2um filter unit.

The other constituents of those culture media used in this project were donor horse serum and foetal calf serum. These sera were stored frozen at -20°C . After removal from the deep freeze the sera were thawed by cold running tap water. Once thawed completely, the sera were placed in a water bath at 56°C and left for 30min. This inactivated the complement proteins originally present in the sera. Sera was then passed through a 0.45um filter [SYBRON, Nalge] collected and passed through a 0.2um filter. At this point the sera could be added to MEM or refrozen in smaller volumes for later use.

The final culture media were produced by mixing the supplemented MEM with sera. Initially the mixture used to maintain the cells was

10% donor horse serum, 10% foetal calf serum and 80% supplemented MEM. This was referred to as 10/10 culture medium. Culture media used after the first week in vitro consisted of 10% donor horse serum and 90% supplemented MEM. This was referred to as 10% culture medium.

All culture media were kept in an incubator [10% carbon dioxide atmosphere, 37°C] to equilibrate for at least 12 hours before use. The tops of bottles containing the culture media were loosened to allow exposure of the contents to the atmosphere inside the incubator without a greater risk of contamination.

COATING OF THE PLASTIC CULTURE DISHES WITH A LAYER OF COLLAGEN

Acid soluble collagen [Calibiochem] from calf skin was dissolved in 100ml of 1:1000 glacial acetic acid which had been filtered through a 0.2um filter unit. The collagen was left to dissolve overnight. The final solution could be stored in a refrigerator for up to 3 months. The following preparation was undertaken in a lamina flow cabinet. A drop of the collagen solution from a Pasteur pipette was placed on the base of a 35mm² plastic culture dish [Sterlin]. The drop was spread evenly over the base using a heat sealed Pasteur pipette. The culture dishes were left in the laminar flow cabinet with their lids in place until required; at least 12 hours.

II.3.1] TRYPSINIZATION

The dissecting medium was removed from the tissue using a Pasteur pipette. A 0.5ml aliquot of 0.25% trypsin solution in dissecting medium was added to the tissue. The culture dish was put into a 37°C incubator with an atmosphere of 10% CO₂ and a humidity of approximately 50%. The tissue remained under these conditions for 30 min.

After the incubation period, the culture dishes were returned to the laminar flow cabinet for the trituration phase.

II.3.2] TRITURATION

Trituration involved dissociation of the enzymatically treated tissue. The tissue was dissociated by passage through a narrow opening, e.g. the orifice of a Pasteur pipette. The force generated by this passage removed the outermost cells from the tissue. This allowed access to the underlying cells during the next passage. Thus, it was possible to gradually dissociate the tissue.

The remaining part of this section describes the complete method used for the trituration of the tissue. A 1ml aliquot of 10/10 culture medium was added to the trypsin solution and tissue. This 1.5ml of solution and tissue were then transferred to a sterile 10ml tube containing a further 1ml of 10/10 culture medium. Approximately 1ml of the tissue and medium mixture was taken up into a Pasteur pipette and expelled back into the tube. This procedure was repeated fifteen times. The small clumps and single cells, which had been

dissociated from the main mass of tissue, were now in suspension.

The remaining large clumps of tissue were allowed to settle for 2 min, leaving the smaller particles suspended above. A 1.5ml aliquot of the suspension was transferred to another tube. This was replaced by 1.5ml of fresh 10/10 culture medium.

To dissociate the remaining tissue a greater disruptive force was required. This was obtained by reducing the diameter of the Pasteur pipette's orifice. The reduction was obtained by passing the orifice through a Bunsen burner flame ["flame-polishing"]. This "flame-polishing" made the glass molten and start to flow, thus partially sealing the tip and reducing the size of the orifice. At each stage of the trituration, the pipette used had a smaller orifice than previously. As a result, an increased force was achieved with each stage thus causing a gradual breakdown of stubborn pieces of tissue. High yields of viable cells were obtained using this technique. Further improvements were made by using an autopipette, [Pipetaid, Metframe/Mattel] in conjunction with the Pasteur pipette; instead of the plastic teat. The autopipette produced constant pressures which reduced variation and over long periods did not tire the operator so much as bulb/teat pipetting.

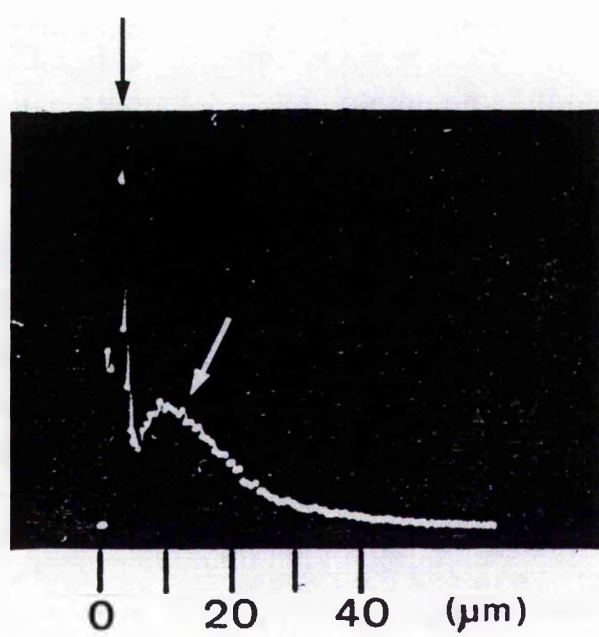
When all of the tissue had been dissociated, the volume of the resulting cell/tissue suspension was increased to 10ml with fresh 10/10 culture medium. The 10ml of cell suspension was then passed through a 45um filter which further dissociated the cells in the suspension. This served to increase the proportion of single cells in the final suspension.

FIGURE II.4.

DISTRIBUTION OF PARTICLES [CELLS] IN THE FINAL
SUSPENSION

This photograph was taken from the oscilloscope screen of the channeliser [Coulter]. It depicted distribution of particle sizes in the sample of diluted cell suspension. Increasing particle size from left to right on the X-axis [microns] and number of particles on the Y-axis.

The large peak on the left [indicated by the arrow] represented the debris from the dissection. This was not included in the cell count. The second peak between 5 and 30 μ m, [white arrow] represented the cellular material.



II.3.3] CELL COUNTING

A 0.5ml aliquot of the 10ml suspension was taken and used to estimate the total number of cells present in the remaining 9.5ml. The 0.5ml was diluted to 20ml with fresh isotonic saline [Isoton] and mixed thoroughly, but gently. A particle counter [Coulter] was used to measure the number of particles [cells] in the suspension with sizes ranging from 5 to 50um in diameter. Distribution of particle sizes was displayed on an accompanying channeliser [Coulter; fig. II.4]. From this the proportion of particles/cells, relative to debris, could be determined. The following calculation was used to determine the number of cells per ml in the original cell suspension.

a= cells per sample used by the cell counter.

x= cells per ml, in the original suspension

y= size of sample used by the cell counter

z= dilution factor [of the sample used by the cell counter]

$$x = [z.a]/y$$

In early studies, Trypan blue was used to check the viability of the cells in the suspension. After 3 months, because the viability had remained constant at approximately 90%; e.g. 90% of the cells excluded the dye, this step was omitted. The volume of the original cell suspension which contained 10^5 viable cells was calculated using the following relationship.

b= viable cells, as a decimal

c= amount of suspension containing 10^5 cells

$$c = x / b.10^5$$

II.3.4] CELL PLATING

The volume of suspension which contained 10^5 cells [c] was aliquoted into 35mm diameter plastic culture dishes [Sterlin]. These culture dishes had previously been coated with collagen [Calibiochem]. Two methods of cell plating were tested.

II.3.4.1] EVEN-DENSITY TECHNIQUE.

Here the cells were plated as above. Fresh 10/10 culture medium was added directly to the aliquot of cell suspension to increase the volume to 1.5 ml. The contents were then completely mixed in the culture dish.

II.3.4.2] CENTRE-SPOT TECHNIQUE.

The highest density of cells were grown in the centre of the culture dish. This was done by placing the aliquot of cell suspension in the centre of the culture dish. Fresh 10/10 culture medium was added around the edge of the dish so as not to disturb the cells from the centre of the culture dish.

The volume in each dish was increased to 1.5ml with 10/10 culture medium. The cells now appeared as in figure II.5a. These dishes were placed inside a plastic tray and transferred to a 37°C incubator with a CO_2 tension of 10% and a humidity of approximately 50%.

FIGURE II.5.

CELLS IN CULTURE

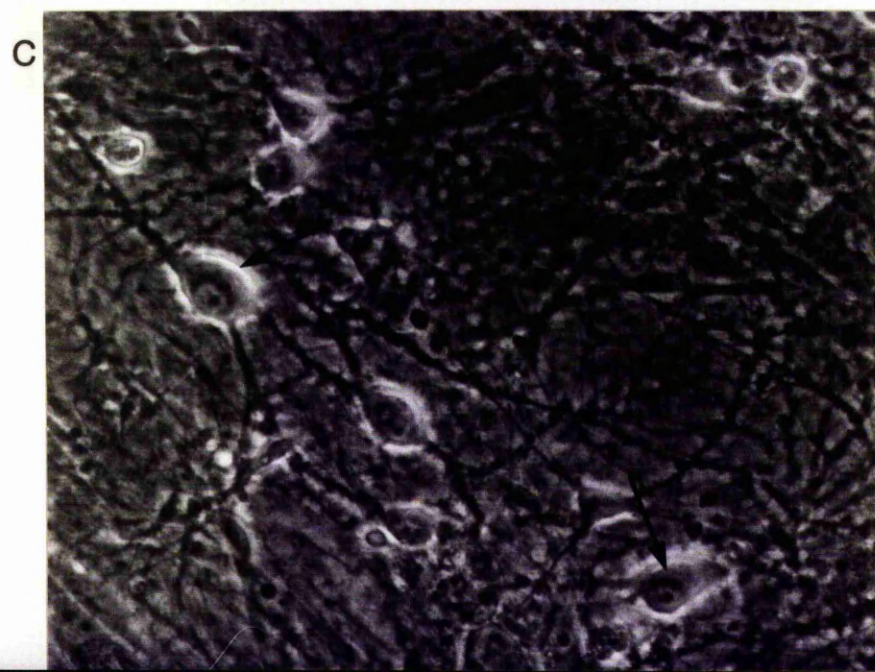
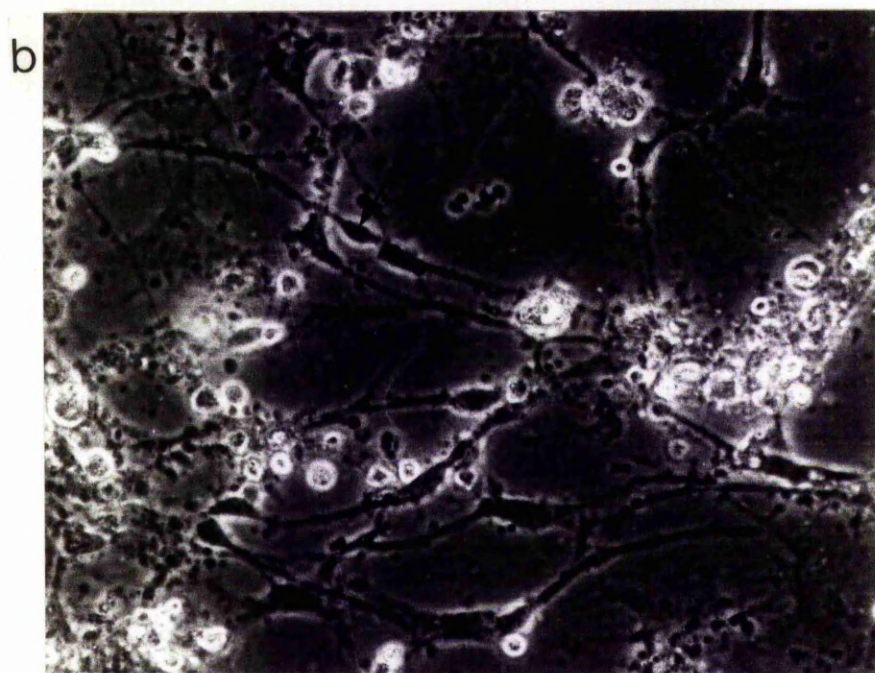
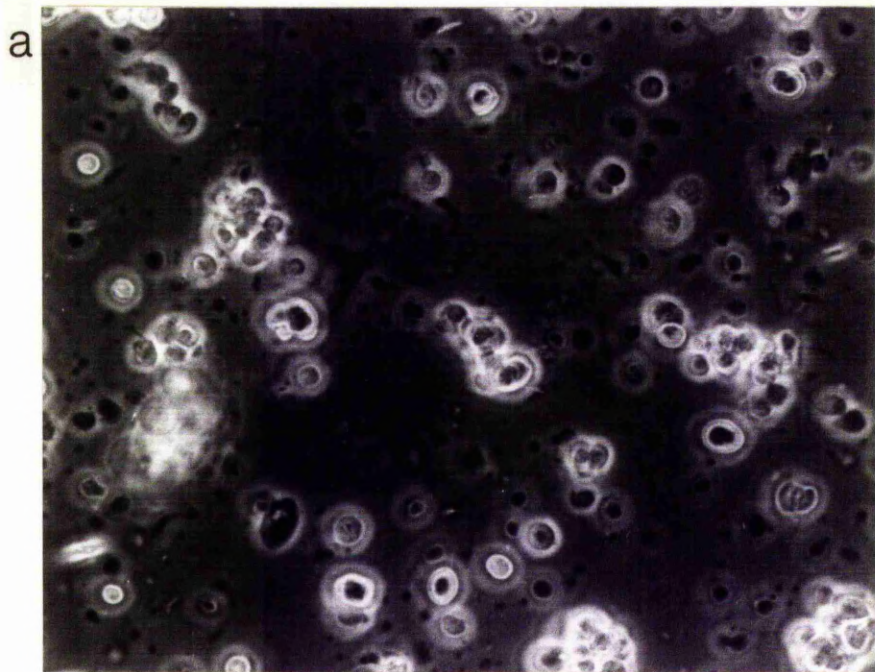
These photomicrographs show cells in culture, at different times after the dissection.

Scale bars represent 30um.

a] Cells one hour after plating into culture dishes. Single and clumped cells were present, however, few processes were visible.

b] After one week in culture, many bipolar cells could be seen. The non-neuronal monolayer was complete.

c] Four weeks in culture. Neuronal cell bodies were distinctive [arrows], most appeared to be multipolar.



II.4] MAINTENANCE OF THE CULTURES

II.4.1] GROWTH IN CULTURE

A visual appraisal of the cells was made after 48 hours in culture. The culture dishes were placed on the stage of an inverted microscope [Nikon] where the cells were viewed under phase contrast illumination at a total magnification 250x. Observations were made of the density, growth rate and confluency of the cell layer. Confluency was indicated by the presence of a complete monolayer of fibroblasts on the base of the culture dish, underlying the neurones. If the cells were not confluent, 0.5ml of the culture medium from each dish was replaced by 0.5ml of fresh 10/10 culture medium. These cells were then checked daily for confluency.

When the cells attained confluency, all the medium in each culture dish was removed and replaced by a solution of 10^{-4} M 5-Fluorodeoxyuridine and 10^{-4} M Uridine [FudR] in 10% culture medium. After two days the 10% culture medium containing FudR was replaced by 10% culture medium without FudR. At this time the cells appeared as in figure II.5b. From this point on, 0.5ml of medium from each culture dish was replaced by fresh 10% culture medium three times a week.

Following the above procedure, cells were maintained in culture for periods in excess of 3 months. However, the average length of time in culture was between 4 and 6 weeks, [fig II.5c]. This was due to the use of the cell cultures for experimentation and/or unforeseen contamination.

FIGURE II.6.

CELL DENSITY

These photomicrographs show the effect that varying the cell density had on the appearance of the cells in culture.

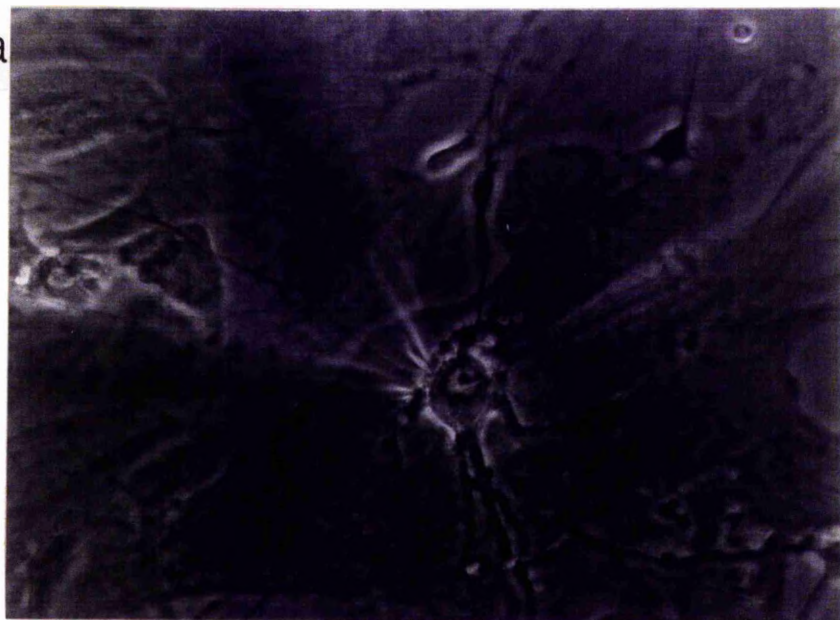
Scale bars represent, 30 μ m in a) and b), and 100 μ m in c).

a] The ideal recording situation. Cell body and processes were clearly visible. The recording electrode entered from the right and the drug application pipettes entered from the left.

b] This culture was overgrown. Too many cells were initially allocated to the culture dish. The result, a high number of non-neuronal cells forming a dense cell-layer on the bottom of the culture dish.

c] Few neurones were present in this [six week old] culture. Those present had extensive processes which were clearly visible. The flattened non-neuronal cells can be clearly seen in this situation.

a



b



c

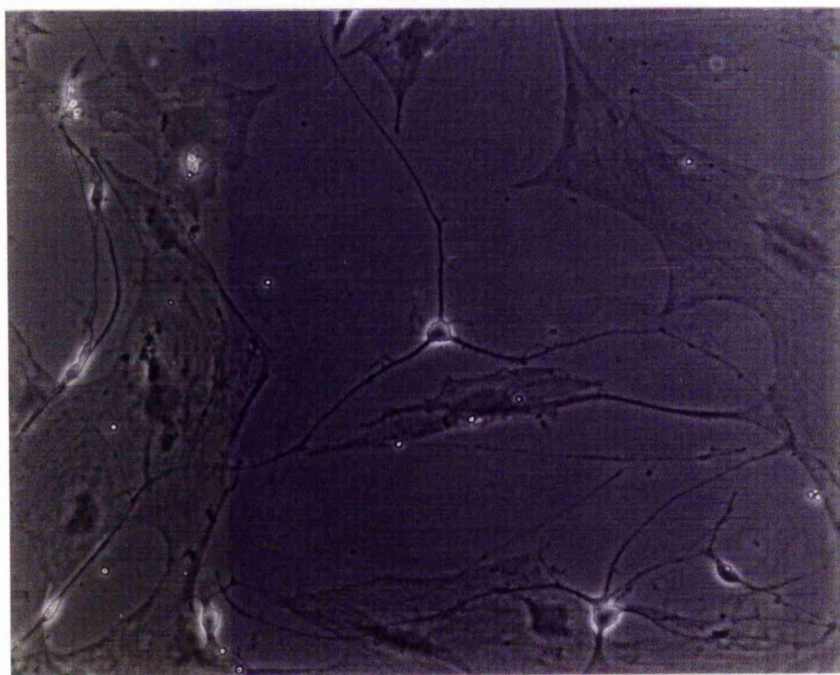


FIGURE II.7.

CONTAMINATION

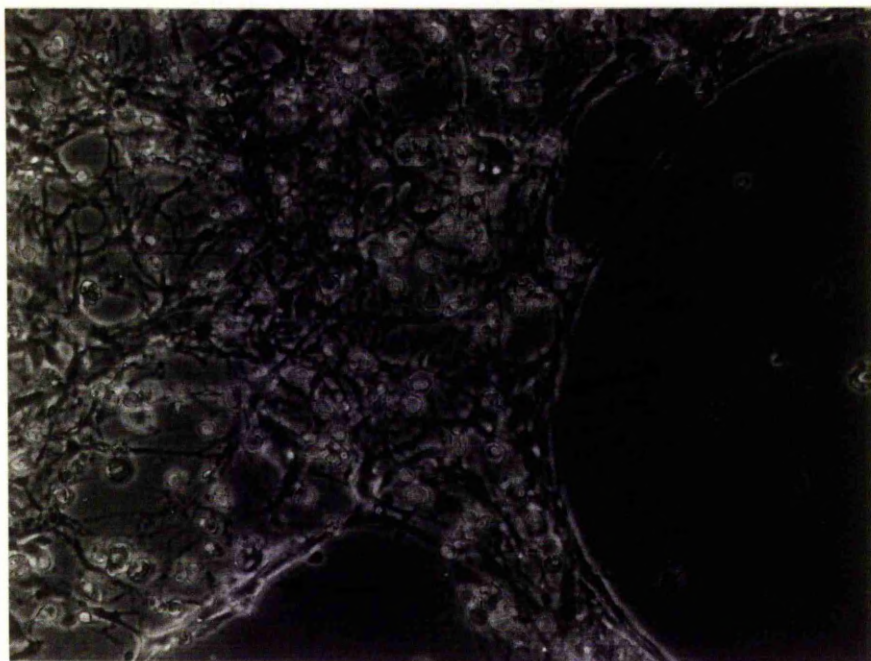
The effects of infection and the appearance of two of the major contaminants. Scale bars represent, a) 100 μ m, b) 50 μ m, c) 20 μ m.

a] The effects of contamination. The cells lose their definite morphologies, becoming phase bright and granular. The cell layer eventually detached from the culture dish, as in the photomicrograph [arrow].

b] Fungal hyphae, these appeared as long strands [photomicrograph] emanating from one locus, these strands were seen to branch at irregular intervals [arrow].

c] Bacterial contamination. Clumps of bacteria [arrows].

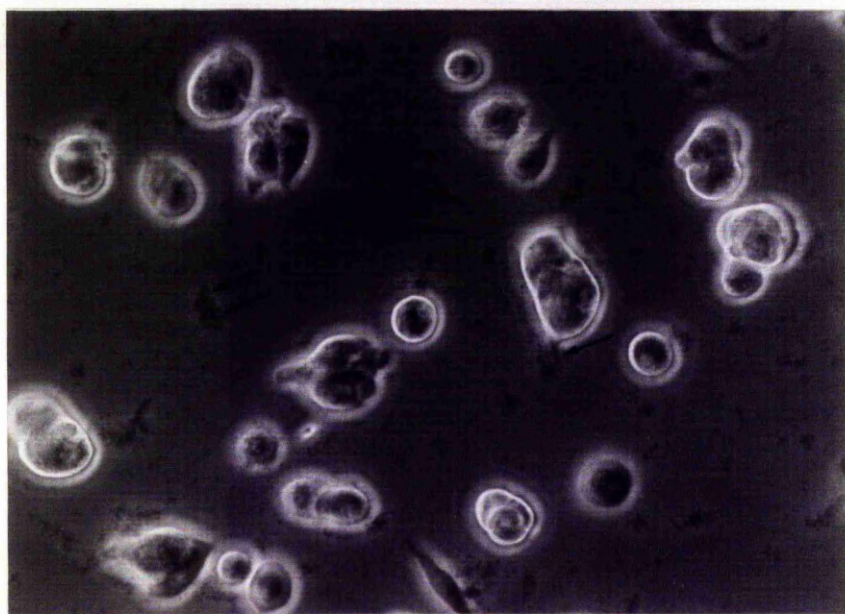
a



b



c



II.4.2] EVALUATION OF THE CULTURES

After the initial growth period the condition of the cultures was checked daily under the microscope. In the evaluation of the cultured cells certain criteria were noted:

- A] general density of the cells
- B] the relative ratio of neurone-like to glial-like and fibroblast cells
- C] the relative ratio of live to moribund cells
- D] the presence of foreign bodies, i.e., contaminations or infections.

These points are illustrated in figures II.6 and II.7, which should be compared with the "normal" culture shown in figure II.5c. Those culture dishes which fell into categories C and D were potentially dangerous to the rest of the cultures as well as the other workers in the laboratory. Consequently, these cultures were either isolated or discarded. Contaminated culture dishes were immersed in a 10% solution of cetricimide [Savlon] in water and left for at least one week.

CHAPTER III

METHODS II

ELECTROPHYSIOLOGY

Electrophysiology is the study of the electrical properties of living organisms, particularly the nervous system. It involves the measurement of electrical potentials and currents generated by cells and tissues. The field is divided into several sub-disciplines, including neurophysiology, cardiac electrophysiology, and cellular electrophysiology. Key techniques used in electrophysiology include patch-clamp recording, voltage-clamp, and current-clamp. These methods allow researchers to study the function of individual cells and the electrical coupling between them. Electrophysiology has been instrumental in understanding the basic principles of neural signaling and the pathophysiology of various neurological disorders.

III.1] ELECTRODES

III.1.1] ELECTRODE-PULLER

Recording electrodes were produced on a moving coil electrode puller of the Ensor design, [Ensor, 1979] supplied by Clarke Electromedical. Certain modifications were made which increased its reliability:

A] Plastic nuts with knurled edges were originally used to tighten the collets. However, the plastic was eroded very quickly, thus making the tightening of the collets increasingly difficult. The resulting lack of control increased the variation in the final electrode and the amount of wasted glass. Consequently, the plastic nuts were replaced by brass replicas.

As an additional feature, small holes were drilled into the knurled edge of the brass nuts. These could accept a small metal bar which gave more control during the tightening of the collets and were less susceptible to erosion.

B] The glass capillaries often slipped from the collets during a pull cycle. The slipping was reduced by applying a resin [SURGRIP] to the teeth of the collets prior to loading the glass.

C] The ends of the glass capillaries scraped the inside of the collets. The resulting flakes of plastic wedged between the collet and glass capillary creating uneven pressures along the glass.

FIGURE III.1.

THE ELECTRODES

These figures show the types of electrode used for intracellular recording and dye-injection during the project.

a] The "tipette".

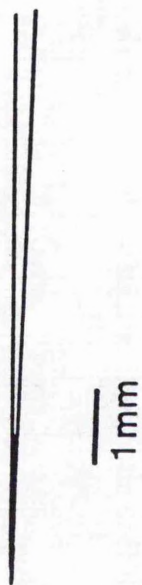
b] The normal shape.

Upper figures show camera lucida representations of the electrodes at low magnification. Lower figures show a scanning electron-micrograph of the electrode tip.

a



b



This usually resulted in breakage of the capillary. The scraping was reduced by "flame-polishing" the ends of the glass before use.

These modifications greatly reduced the variation between electrodes and the percentage of breakages produced by the puller.

III.1.2] RECORDING-ELECTRODES

At the start of the project, attempts were made to produce electrodes from borosilicate glass capillaries. Both thin and thick walled capillaries of external diameters 1, 1.2, and 1.5mm were used in the making of the electrodes. The better electrodes were obtained using 1.5mm, thin walled glass capillaries, [Clarke Electromedical, GC150-TF-15]. These capillaries contained a glass filament which allowed the finished electrode to be filled easily with electrolyte.

Improvements were made to the design of the "standard" intracellular electrode so that it could be used more efficiently in conjunction with cultured neurones. The design ["Tipette", fig III.1a] was based on the requirements for an electrode which could be used repeatedly with little decrement to the quality of impalement. Another factor, which had to be taken into account, was the distortion caused to the meniscus when the electrode was in recording medium and the corresponding changes in quality of the phase contrast image. However, compensations made for these factors tended to increase the resistance of the final electrode and therefore, compromises had to be made.

The final shape consisted of an acutely tapered barrel, the shank was pulled very sharply to produce a prolonged tip terminating in a "filament". The final 5um of tip was observed using an electron-microscope. This showed that there was very little taper over this distance and that the external diameter was between 0.1 and 0.3um [fig III.1a]. It appeared as though the "Tipette" could be broken back for up to 5um with very little reduction in tip diameter, whereas the "standard" electrode was shown to have a 2 to 3 fold increase in diameter over a similar distance from the tip [fig III.1b].

"Tipettes" could only be used for intracellular recording from cultured cells. The limiting factor appeared to be the fragility of the tip especially when "Tipettes" were used in a preparation where connective tissue had to be penetrated, [e.g. a brain slice, Blaxter, T.J. personal communication]. However, little or no connective tissue overlies the cells in culture, [Ransom et al., 1977a] and it was found that the "Tipette" could be used repetitively, [up to 6 stable impalements].

The electrodes were checked under a microscope using light-field illumination, at a total magnification of either x250 or x400. If the electrodes appeared satisfactory they were stored in dry petri-dishes until required. The electrodes were filled with an electrolyte and stored in a humid atmosphere for between 15 min and 24 hours directly before being used. The electrolyte was either 2M potassium chloride or 2M potassium acetate solution made in distilled water and filtered through a 0.2um millipore filter.

Stable impalements, up to 7.5 hours, were obtained using electrodes based on the "Tipette" design.

III.1.3] PIPETTES FOR DRUG APPLICATION

Pressure ejection and iontophoretic pipettes were manufactured from 1.5mm capillary glass, [GC150TF-15] on a homemade vertical electrode puller. When these electrodes were filled with 2M potassium chloride they had resistances of between 2 and 15 Megaohms.

The shape of these electrodes was determined by 2 factors.

A] As with the recording electrodes, the shank was long and thin. This reduced the distortion of the meniscus. Consequently, the phase contrast optics remained of a reasonable quality.

B] The distance between the condenser and the culture dish was small [circa 3-5 cm] and restricted the manoeuvrability of a straight electrode. To accommodate this, the end section was bent at an angle of approximately 75° to the shaft.

III.2] RECORDING SYSTEM

III.2.1] ELECTRONIC APPARATUS FOR INTRACELLULAR RECORDING

A single electrode current clamp system was used. The signal from the recording electrode was picked up by a non-polarizing silver chloride coated silver wire which was in contact with the electrolyte in the recording electrode. This was connected via a probe, to a preamplifier [Neurolog NL102, Digitimer]. A reference was taken from the culture dish to the preamplifier, via an agar bridge. Wherever possible, all wires were screened with earthed wire to help reduce electrically induced noise.

Output from the preamplifier [100x the input from the electrode] was displayed on an oscilloscope [a Tektronix 5113 dual beam oscilloscope with a 5A12N differential amplifier]. A permanent record of required data was taken using a Brush 220 series pen recorder, [Gould]. The signal for this operation came from a one to one output at the rear of the oscilloscope.

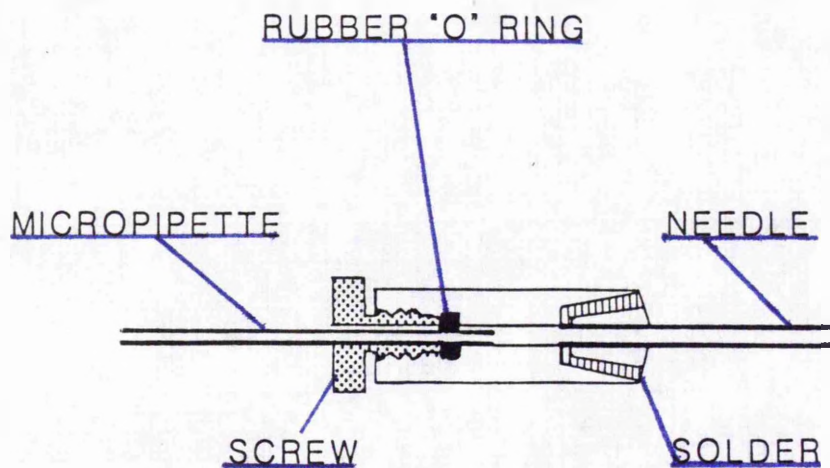
The stimulator unit, consisting of a power supply, a pulse generator and a waveform generator [Tektronix, model No.s 160A, 161 and 162, respectively], was used to generate square pulses of constant voltage. These were injected into the preamplifier where they were converted into constant current pulses. The constant current pulses were passed through the electrode and used in the monitoring of membrane resistance and balancing the electrode resistance out of the circuit. The amount of voltage input to the

FIGURE III.2.

ELECTRODE HOLDER FOR THE DRUG PIPETTES

A cross-section through one of the home-made pressure-tight, electrode holders. These were used predominantly for drug application by pressure-ejection [pico-spritzing]. It was made from a metal 21 gauge needle [luer-lock] and a piece of brass. The electrode was fastened in place by tightening the screw. This action compressed the rubber "o" ring around the micropipette making it pressure tight at pressures of up to 45psi.

Scale bar = 1cm.



preamplifier determined the current output to the electrode.

III.3] DRUG APPLICATION

The drug containing solutions for application by iontophoresis were prepared in distilled water. Those drug solutions which were to be pressure ejected were prepared in recording medium [either the normal or the high magnesium, dependent upon which was originally bathing the cells]. When the medium bathing the cells was changed for one with a different variety of ionic concentrations, an adjacent pressure ejection pipette was used to apply recording medium alone. The small quantities ejected resulted in little or no voltage artefact, otherwise a new series of pressure ejection pipettes were made.

III.3.1] PRESSURE-EJECTION

A Picospritzer II [General valve corporation] was used to regulate the pressure and duration of the pulses used. The system was pressurised by hand using a Killaspray cadet 4 [ASL Airflow Ltd.] An air-line from the picospritzer passed through a home-made single-input multi-output tap whose outputs were connected to pressure tight home-made electrode holders, [fig III.2].

III.3.2] IONTOPHORESIS

A battery driven micro-iontophoresis programmer [model 160, WPI Instruments] was used to retain and expel required amounts of drug from the electrode. The circuit included a connection from the iontophoresis programmer to the solution in the electrode via a non-polarizing silver chloride coated silver wire. The recording chamber was connected to the iontophoresis programmer via an agar bridge containing 1M sodium chloride.

III.4] PERFUSION SYSTEM

Tubing was joined together by sterile needles [18 gauge, B-D Plastics] which were secured in place with Araldite. Portex and flexible silicon tubing was used which had an external diameter of between 2 and 5mm.

Sterile equipment was used in the assembly of the perfusion system. After assembly, the system was thoroughly washed through with hot water to remove any impurities which may otherwise have interfered with experiments. Each section was washed out with hot water every day, especially before and after experimentation. The reservoirs were fitted with caps which prevented the introduction of infectious material [dust]. Using these precautions the system remained relatively sterile, even so, every month the whole system was totally renewed. The perfusion system was composed of 2 separate sections.

FIGURE III.3.

THE PERFUSION SYSTEM

a] The full perfusion system, [lengths of tubing not to scale].

b] The inset shows how the medium uptake worked. The fluctuation in depth was dependent upon the angle at which the bevelled tip of the needle was immersed.

Scale bar= 1mm

Abbreviations used in the diagram:

Bt- Bubble trap, a 5ml syringe [B-D plastics]

CD- Culture dish

Dp- Delta pump [Schuco]

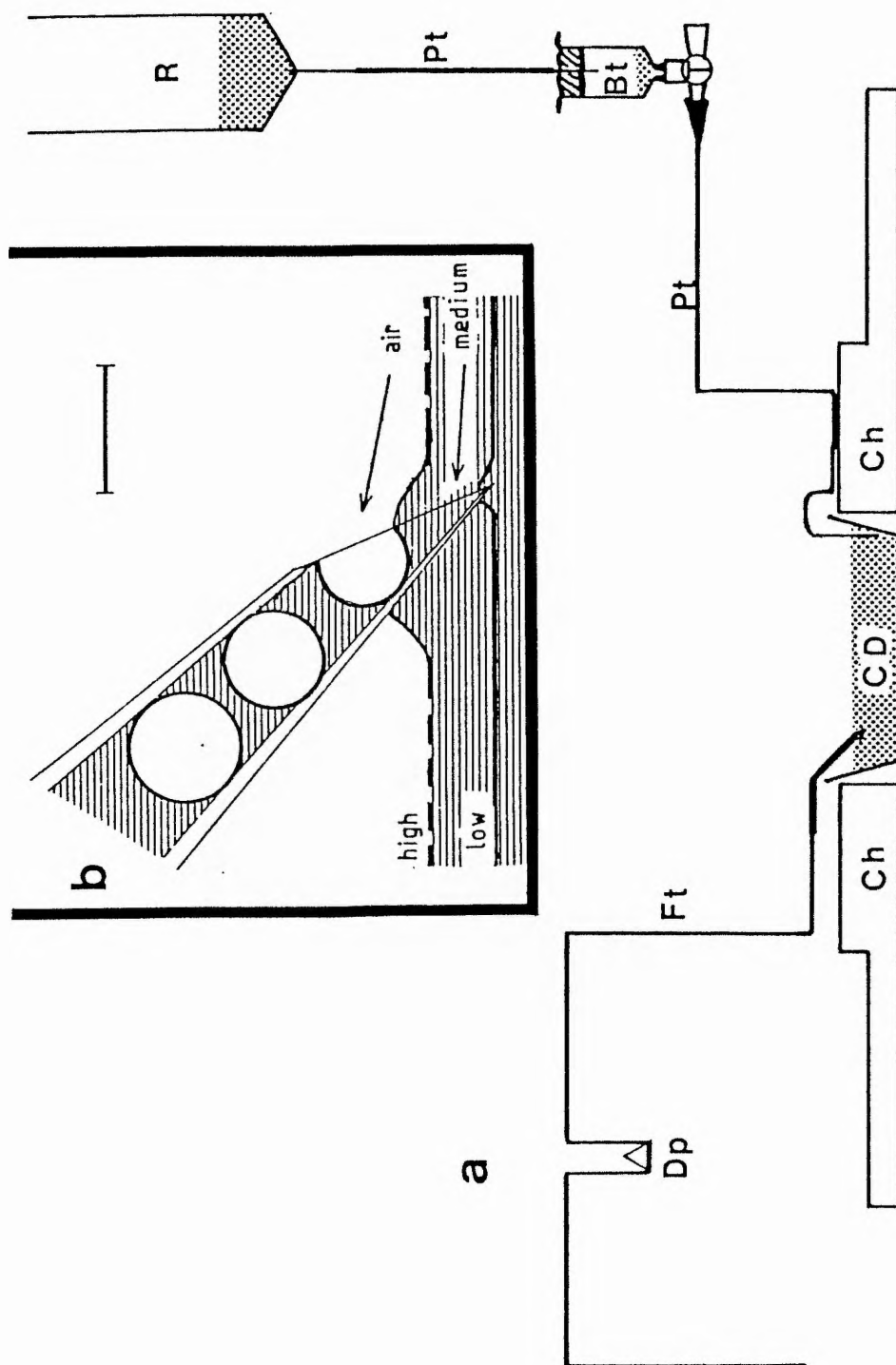
PT- Portex tubing

R - Reservoir, a 50ml conical tube

Ch- Recording chamber, a perspex ring [details as for fig III.5]

Ft- Flexible silicon tubing [i.d. 4mm]

T -Tap, used to select which medium perfused the dish; usually there were two reservoirs which fed into the tap.



III.4.1] INPUT SECTION

The input system consisted of a passive flow from a reservoir into the culture dish. The reservoir was a 50ml plastic culture tube [Sterlin] which was linked by Portex tubing to a bubble trap, made from a 5ml plastic syringe [B-D plastics]. Output from this syringe passed through a plastic three-way tap [Vygon], as shown in figure III.3a, and along Portex tubing which stretched from the tap to the recording chamber. The tubing terminated in a 23 gauge needle [B-D plastics] shaped as in figure III.3a. This needle allowed fresh recording medium to enter the recording chamber from under the surface of the existing recording medium and towards the side of the culture dish. In this way, disturbance of the cell layer was minimized.

III.4.2] OUTPUT SECTION

A delta pump [Schuco] was used in the active removal of solution from the culture dish. A 19 gauge needle [B-D plastics] was bent and positioned as in figure III.3a. This needle was connected to silicon tubing [i.d. \geq 3mm] which was continuous with the waste bottle, via the delta pump. The surface of the solution could be set at any required level by adjusting the position of the outflow needle. At no time was the solution in the culture dish continuous with that in the waste bottle.

III.4.3] PERFUSION

Whilst the system was operating, there was a steady input of fresh solution at a rate of between 0.5 and 1.0 ml per min. The rate of removal was at least 1.5 times greater than this, causing an uptake of air as well as solution, [fig III.3b]. The resulting flow rate through the system was dependent upon the rate of inflow.

Large variations in the depth of the medium were generated when broken pipettes were used to remove solution from the culture dish. These variations caused removal of solution after the level had dropped well below that of the tip of the pipette. This effect was due to the meniscus of the recording medium. A bevelled tip was found not to hold the meniscus as long as a jagged or broken tip. Therefore, the fluctuation in depth of the solution was correspondingly reduced by using a pipette with a bevelled tip as a suction pipe.

Instead of bevelling a glass pipette, which would break easily and be difficult to unblock, metal syringe needles were used. Conveniently, syringe needles already had bevelled tips. The larger the bore of the needle meant less chance of it blocking. However, this also produced a greater amount of fluctuation. The final choice was a 19 gauge needle [B-D plastics].

Care had to be taken when positioning the outflow needle to determine the depth of the medium. If the needle was placed too close to the edge of the dish there resulted a continual removal of

the recording medium. This was because the meniscus at the edge of the dish curved upwards. The effect allowed the solution to fall to a lower level in the middle of the culture dish before the surface at the edge dropped below the required level.

The time required for a complete change of recording solutions was found by perfusing distilled water into a culture dish containing 0.1M potassium permanganate or vica versa. Potassium permanganate was used because of the colour, which was visible even at low concentrations. The changeover time varied slightly between input systems, usually being between 5 and 10 min. The pattern of flow was also found using this method.

III.5] OPTICAL SYSTEM

The perspex recording chamber and culture dish were positioned as in Figure III.4, on the stage of an inverted microscope [Zeiss IM 35]. The cells were viewed under phase contrast illumination, at a total magnification of either x125 or x312.5. The lowest magnification was used to locate and position the electrodes. Fine positioning of the electrodes, with respect to a cell, impaling and drug application were all monitored at the higher magnification, [fig II.6a].

FIGURE III.4.

PHOTOGRAPH OF THE MICROSCOPE STAGE

Photograph of the recording chamber with the central culture dish [arrow] as positioned on the microscope stage. Electrodes passed under the condenser and into the culture dish.

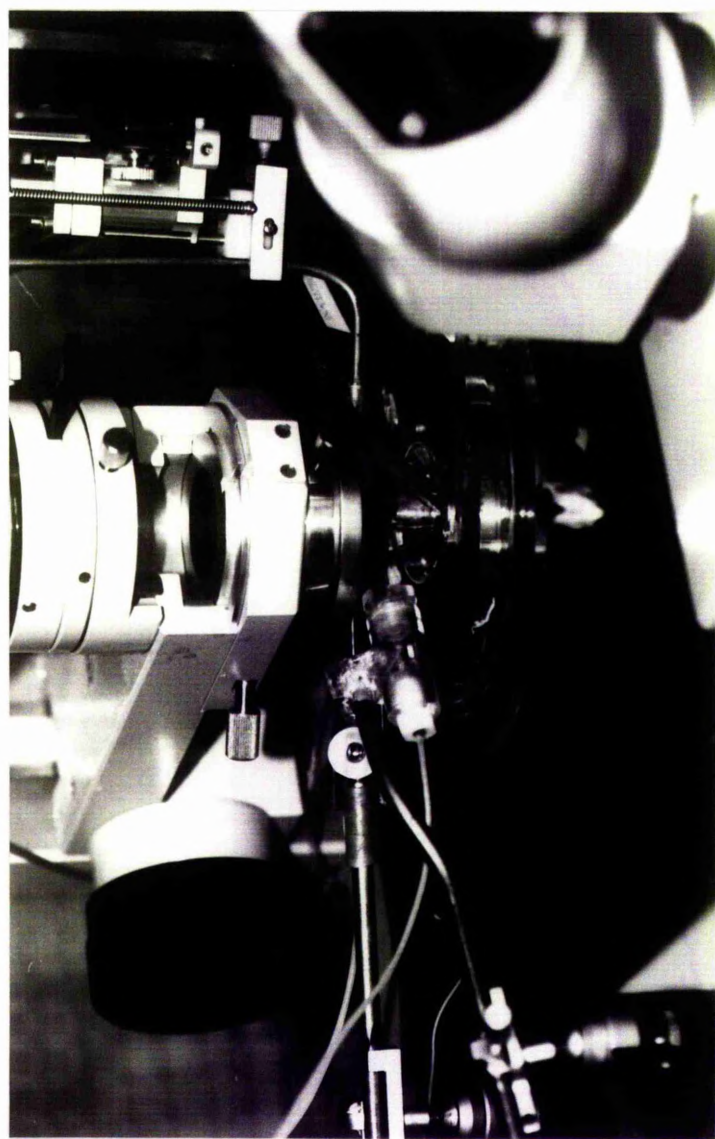


FIGURE III.5.

RECORDING CHAMBER

These figures show a schematic representation of the recording chamber and associated attachments. The scale is 1:1.

a] From above; the agar bridge arrangement and relative positions of the electrodes is shown.

b] Cross section through the chamber from A to B as seen from the side.

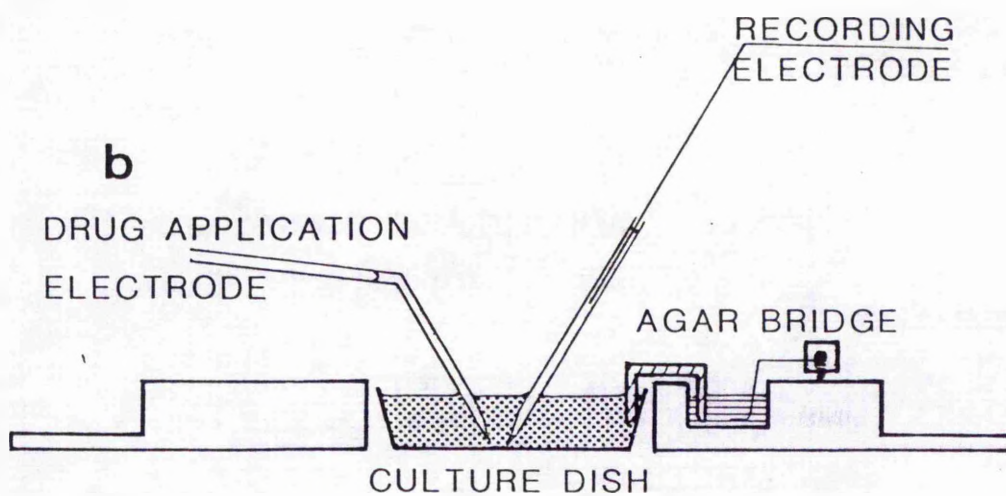
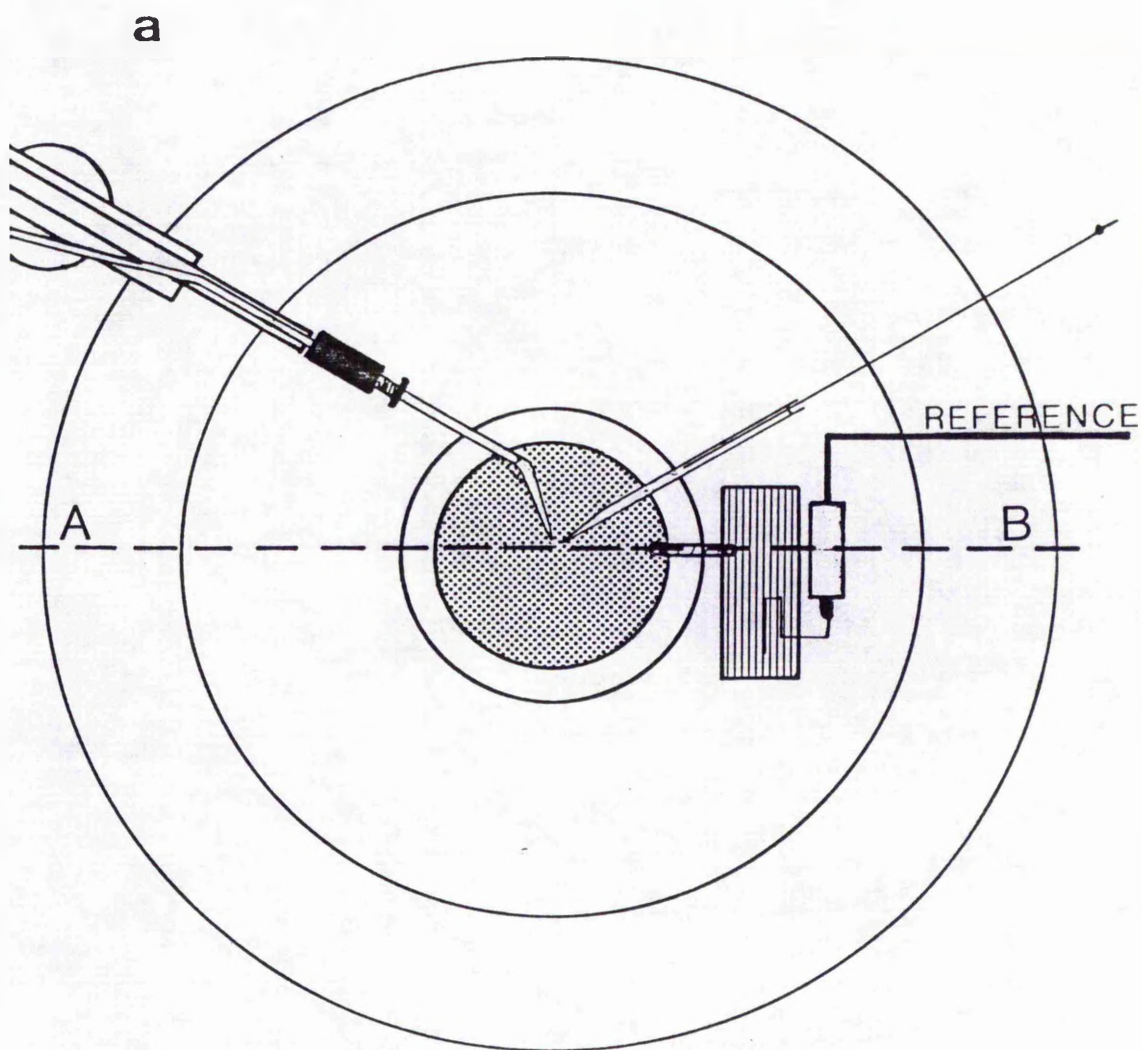
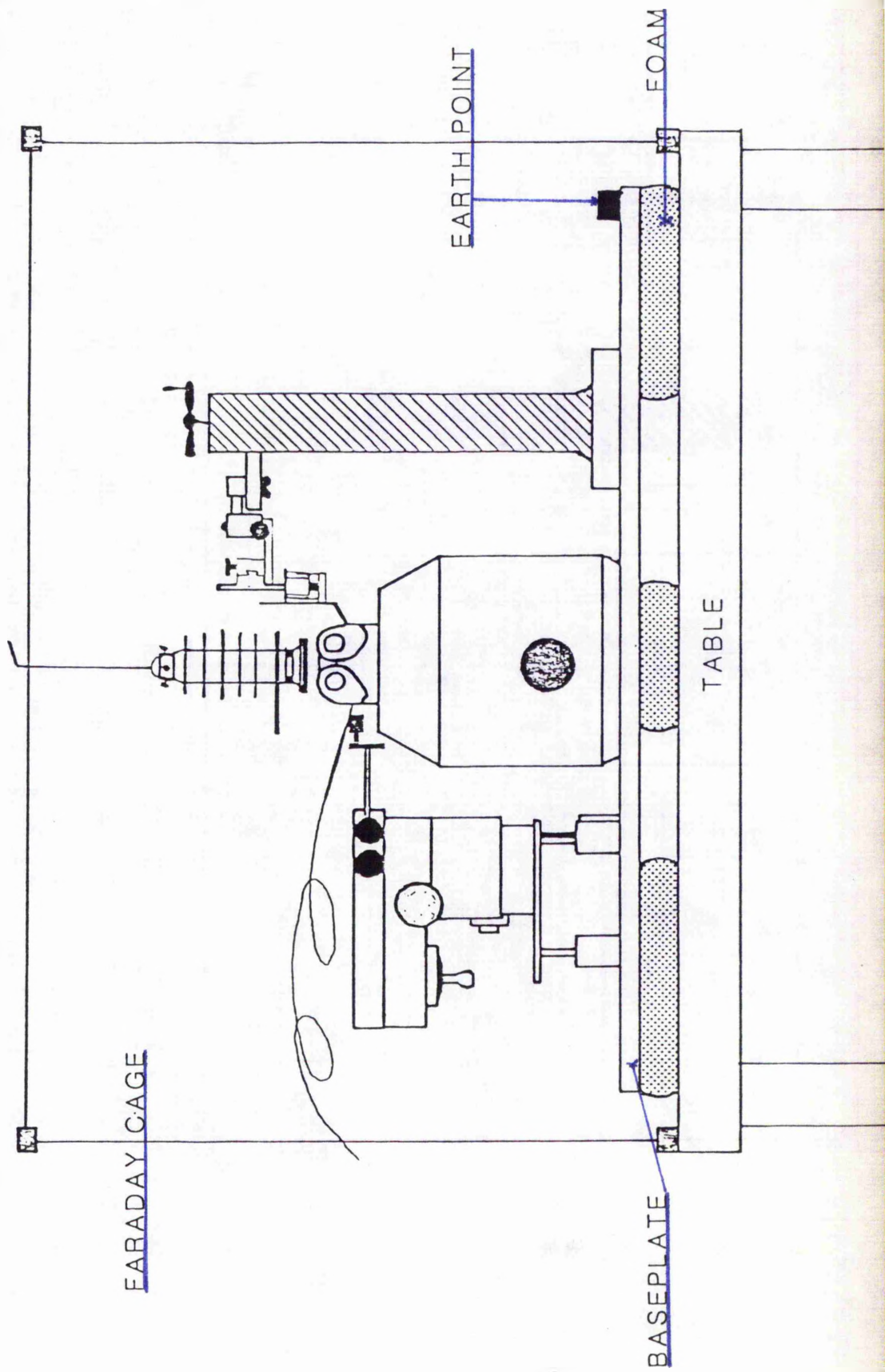


FIGURE III.6.

ARRANGEMENT FOR ELECTROPHYSIOLOGY

This shows the organization of the apparatus [micromanipulators] for recording [right] and drug application [left]. The microscope was central.

The base-plate rested on a layer of foam on the table top. The foam reduced the transmission of mechanical vibrations from the surroundings to the recording chamber. Surrounding the whole system was a Faraday cage resting on the table top, this prevented electrical noise from interfering with the intracellular recordings.



III.5.1] PHOTOMICROGRAPHY

Photomicrographs were taken using the inverted microscope [Zeiss IM 35] and a 35mm camera body [Contax]. A remote shutter release was used to reduce the vibration caused by the manual operation of the camera. The camera body contained an automatic exposure control, which was sufficiently accurate. Photomicrographs were taken using either Pan F or Tri-X film. All films were developed in Aculux [Paterson] and printed on Ilfospeed paper [Ilford]. The vibration associated with the shutter opening limited the use of the camera to the recording of only pre- or post-impaled cells, as in figure II.6a.

III.6] ARRANGEMENT ON THE BASE-PLATE

III.6.1] RECORDING SYSTEM

The recording electrode was supported by a perspex holder and held in place with plasticine [Galtoys]. The electrode holder was connected to a hydraulic microdrive unit, [Kopf] which gave fine vertical movement. This microdrive had a remote control unit which was positioned outside the base-plate. Gross lateral positioning of the recording electrode was provided by a Narishige [5044] micromanipulator, fixed to the microdrive. The micromanipulator was fastened to a Palmer stand. This provided a rigid support and gross vertical movement. The Neurolog headstage unit [Digitimer] was positioned so that the non-polarizing silver chloride coated silver wire, which connected it to the recording electrode, was as short as possible. This latter feature helped minimize the electrical interference [noise].

III.6.2] DRUG APPLICATION SYSTEM

The pressure ejection and iontophoretic electrodes were supported by pressure-tight holders, [fig III.2] on the left of the microscope. Leitz micromanipulators manoeuvred these electrodes in the vertical and horizontal planes. These micromanipulators allowed fine movement of the drug pipettes, under direct visual guidance, with little vibration or drift.

III.6.3] MICROSCOPE

The inverted microscope [Zeiss IM 35] was positioned between the recording system [on the right] and the drug application system [on the left], as in figure III.6. The input section of the perfusion system entered from the right, the output from the left. Most of the wires passed under the microscope, the only one above the microscope stage being a screened DC cable for the microscope lamp. The microscope also functioned as a major part of the electrical screening for the recording system.

III.6.4] MICROSCOPE STAGE

The recording chamber was a perspex ring [fig III.5a], which had facilities for an agar bridge and the perfusion system. This was fixed by plastic screws to a greaseplate, on the microscope stage. The greaseplate allowed fine movement in any 2-dimensional direction. Culture dishes were placed in the centre of the perspex ring, over the objective lens. Electrodes for recording and drug application passed over the recording chamber and into the recording medium in

the culture dish, [fig III.5b].

III.6.5] THE BASE-PLATE

The base-plate was a piece of iron with the dimensions 2x100x70cm. It was used as a common earthing point for all the equipment. The weight of the base-plate increased the stability of the equipment which it supported. A cushion of packing foam was in place between the base-plate and the table to reduce the transmission of mechanical vibrations. This foam damped most of the vibrations passed up from the floor. Figure III.6 shows the position of the base-plate with respect to the rest of the equipment.

III.6.6] THE FARADAY CAGE

The function of this cage was to substantially reduce the reception of electrical noise from the surroundings. An incomplete Faraday cage surrounded the equipment on the base-plate. The cage consisted of a rectangular Dexian framework, of which 3 sides and the top were covered with a metal wire-mesh. The whole cage was electrically continuous and earthed to the base-plate.

III.6.7] EARTHING

All the equipment was connected to a single earth-point on the base-plate using low resistance wire. The base-plate was connected directly to an earth wire from the mains supply. All electronic equipment was disconnected from the mains earth via their plugs and their chassis were reconnected to earth via the base-plate. This

TABLE III.1

MEDIA USED DURING INTRACELLULAR RECORDING

<u>CHEMICAL COMPOUND</u>	<u>MOLARITY [mM]</u>	
	<u>NORMAL</u>	<u>HIGH MAGNESIUM</u>
KCl	5.36	5.36
KH_2PO_4	0.44	0.44
NaCl	136.89	127.89
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.36	0.36
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.00	10.00
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.00	1.00
glucose	10.60	10.60
HEPES	25.00	25.00

MEDIA CONTAINING LOW SODIUM OR LOW CHLORIDE
CONCENTRATIONS

These contained equimolar concentrations of choline chloride [substituted for sodium chloride] or sodium isethionate [substituted for sodium chloride].

MEDIA CONTAINING ALTERED POTASSIUM CONCENTRATIONS

In these, potassium chloride was either replaced with, or substituted for, sodium chloride in an equimolar ratio.

prevented the formation of earth loops. The electrical noise was usually between 100 and 200uV for an electrode of 70Megaohms resistance.

III.7] INTRACELLULAR RECORDING

All intracellular recordings were made at room temperature, between 18° and 23°C, in recording medium [table III.1].

III.7.1] POSITIONING OF THE RECORDING ELECTRODE

The electrode was located under low magnification [x125] prior to entry into the recording medium. It was then lowered to within 100um of the cell layer under direct visual control. The higher magnification [x312.5] was used, in conjunction with the microdrive, to position the electrode close to the perikaryon of a cell. Fine positioning of the perikaryon under the electrode tip was performed using the greaseplate.

III.7.2] MEASUREMENT OF ELECTRODE RESISTANCE

The following measurements were made when the tip of the electrode had entered the recording medium. Constant current pulses were injected through the electrode at 1Hz. The resulting voltage deflections were monitored on the oscilloscope. These deflections were reduced by balancing the resistance of the electrode against that of the bridge circuit in the preamplifier. When in balance no voltage deflections could be seen. The electrode resistance was read directly off the bridge balance dial on the preamplifier. If for any

reason the electrode resistance changed, the circuit became unbalanced and consequentially, the voltage deflections reappeared.

An independent method of checking the calibration of the bridge balance unit was also available. The preamplifier was capable of passing a series of 1nA pulses directly through the electrode, by-passing the bridge circuit. The corresponding voltage deflections could be used to calculate the circuit [electrode] resistance.

III.7.3] IMPALEMENT OF A CELL

When the electrode tip came into contact with the cell the electrode resistance increased. This caused the reappearance of the voltage deflections which were being produced by the constant current pulses. Coincident with these electrical events an inflection was usually visible on the cell body. The cell was then impaled by applying a negative current pulse [$< 500\text{ms}$, between ± 10 and $\pm 100\text{nA}$] to the electrode.

Once the electrode tip was inside the cell, the apparent resistance of the electrode fell to approximately that measured in solution. At this point, a membrane potential and occasionally action potentials were seen. However, neither of these were of the "normal" resting size. Eventually, the membrane potential became more negative and the action potentials larger with a shorter duration. This was usually helped by applying a mild hyperpolarizing current [circa -0.4nA] to the inside of the cell, through the electrode. A stable impalement was rarely achieved within 2 min, instead, this usually took up to 15 min.

III.7.4] EVALUATION OF ELECTRICAL PROPERTIES OF THE CELLS

Before any neurone was tested with drug solution, the electrical properties of that neurone were determined.

The following measurements were made:

- A] Action potential duration
- B] Action potential amplitude
- C] Resting membrane potential
- D] Membrane resistance, where possible

Parameters A, B and C were taken for all cells whose impalement lasted for longer than 15 min, and which were capable of firing action potentials on injection of positive current into the cell body.

Membrane resistance measurements and current/voltage relationships were made from cells impaled with balanced electrodes. Those electrodes did not show any voltage deflections when a current of $\pm 0.5\text{nA}$ was passed through them either before or after the impalement.

III.7.5] MEMBRANE RESISTANCE

Membrane resistance was calculated by passing a current of $\pm 0.3\text{nA}$ through the electrode, measuring the corresponding voltage deflections and applying Ohms' law. All voltage deflections were allowed to plateau before being measured. Current-voltage relationships were constructed in a similar manner. The current

injection was either in steps or by varying the size of the individual current pulses. Current steps were either ± 0.1 or ± 0.2 nA.

III.7.6] ASSESSMENT OF THE ELECTRICAL PROPERTIES OF ELECTRODES DURING AN IMPALEMENT

The electrical properties of the electrode often determined how useful it would be during an impalement. Problems were caused by: too high an electrode resistance, a noticeably high capacitance or, rectification. All of these properties appeared to be linked. Occasionally, the electrode would block during the impalement. Attempts would be made to clear the blockage, if these were unsuccessful the impalement was terminated and the electrode discarded.

III.8] EXPERIMENTAL PROTOCOL

Once a stable impalement had been obtained and the electrical parameters measured, the cell was categorized as either a dorsal root ganglion neurone, a spinal neurone, a glial cell or a muscle cell.

Primary interest lay with the spinal neurones. Therefore, after ascertaining the cell was a spinal neurone, the following protocol was used:

- 1] Test the neurone with peptide at various membrane potentials. These included especially the potentials more positive than membrane potential.
- 2] If no response was seen either:
 - a] test drug on the surrounding cells, noting any variation in synaptic activity recorded from the impaled neurone.
 - b] test effect of the peptide on an amino acid response from the impaled neurone.
 - c] withdraw the recording electrode.
- 3] If the neurone responded to peptide application:
 - a] check for artifacts by varying the membrane potential of the impaled neurone.
 - b] vary dose and area of peptide administration.
 - c] determine reversal potential in normal medium and high magnesium medium. Note any voltage dependence or desensitization.
 - d] change the ionic composition of the medium [with respect to sodium, chloride or potassium].
- 4] When adding an assumed antagonist or "modulator":
 - a] Obtain repeatable responses to the agonist using a set dose cycle.
 - b] Add antagonist prior to a dose of agonist in the cycle. Do not add agonist and antagonist simultaneously.

III.9] LUCIFER DYE INJECTIONS

III.9.1] PREPARATION OF THE DYE

Lucifer yellow CH [Stewart, 1981] was dissolved in a 1M lithium chloride solution at a concentration of 30mg/ml.

III.9.2] ELECTRODES

Electrodes of the "standard" design were used for the intracellular dye injection, [fig III.1b]. The resistance of the electrode, which gave an indication of the tip diameter, was required to be as low as possible when filled with the dye solution [circa 40 Megaohms].

Care was taken not to add any impurities to the electrolyte/dye solution while filling the electrode. Contamination of the dye containing electrolyte increased the possibility of the electrode tip blocking when current was passed through it. If the electrode was blocked, little or no dye would be ejected. Therefore, when sufficient dye was available, the final electrolyte/dye solution was filtered through a 0.2um filter unit [Millipore] before use.

III.9.3] DYE INJECTION

The expected quality of impalement was the same as that obtained during an intracellular recording experiment. Once a cell was impaled, the light source was switched off and the room lights dimmed. Dye injection proceeded using one of two methods: either a continual current injection of approximately -0.5nA or, 200ms duration pulses of current $[-0.5\text{nA}]$ at a frequency of 1Hz. The period of dye injection was between 15 and 45 min. Only one cell per $100\mu\text{m}^2$ of culture dish was injected with dye.

Successful dye injection depended upon the cells remaining alive for at least 4 hours post-impalement. Therefore, the electrode was removed carefully from the injected cell, causing as little damage as possible. After immersion in fresh recording medium the cells were then left in the dark for between 5 and 10 hours, at room temperature. This was a precaution because the exposure of the dye filled cells to intense lighting could have damaged them. This would be caused by the photo-oxidation of lucifer yellow.

III.9.4] PROCESSING

Processing of the culture, after dye injection, started with a 5 min fixation in a freshly made 1% solution of paraformaldehyde in phosphate buffer at pH 7.4. After fixation, the cells were dehydrated using a progression of alcohols, [alcohol strengths 20%, 50%, 75%, 90% and two of 100%, 5 min in each]. The cell layer was mounted in glycerol and covered with a coverslip, which kept out moisture. The cells were then visualized using a microscope [Leitz] set for phase contrast illumination. This microscope also had

facilities for ultraviolet illumination. The fluorescence was viewed at 530 nm, using a 280 nm excitation filter. Photomicrographs of the neurone were made under phase contrast and/or fluorescence using a camera body [Leica] attached to the microscope. Tri-X film was used and developed as before. Line drawings were made from the microscope and corrected with reference to the photographic negatives.

CHAPTER IV

RESULTS I

GENERAL PROPERTIES OF THE CULTURED CELLS

IV.1] PRODUCTION OF THE CULTURES

IV.1.1] CHANGES OF THE METHOD AND THEIR EFFECT ON THE YIELD OF VIABLE CELLS

The percentage of viable cells in the final suspension depended on a variety of factors. The more important factors are listed below along with the precautions taken to ensure a maximum yield.

A] Transfer of alcohol into the dissecting medium by instruments during the dissection. This was reduced by shaking the alcohol off the instruments after they were removed from the 70% alcohol and before they were used in the preparation of the culture.

B] During trituration, the variation in force produced using a rubber teat on a Pasteur pipette lead to excessive damage of the dissociated cells. This resulted in a lower yield. Another problem associated with this method was the high degree of operator fatigue. Therefore, an autopipette was exchanged for the rubber teat. This autopipette produced constant pressures which gave greater control of the rate of dissociation and was easier to use.

C] The filtration of the cell suspension through a 45um mesh prior to plating out the cells was omitted. This stage was originally used to break up the small clumps of cells thus, increasing the percentage of single cells. However, it was possible that larger cells were being removed by the filter. To test this, the cell suspension was divided into two equal aliquots and only one

of these filtered. Both were then cultured separately. A comparison was made between the cells in each of the cultures. No consistent differences were seen in either morphology, pharmacology or electrophysiology of the cells. This stage was omitted because it had no apparent benefit.

D] The number of available neurones per culture dish was orders of magnitude greater than that required for a single series of experiments. Originally, the culture dishes were plated out using only the even-density technique. This technique wasted over 50% of the cells produced by the dissection. These "extra" cells accumulated around the edge of the culture dish. As any cell which was further than 0.75cm away from the centre of the culture dish could not be impaled with a microelectrode, these "extra" cells were not available for use in this study. When using the even-density method there was a need to obtain the correct density of cells for each culture dish. If too many or too few cells were transferred into a culture dish, the resulting density would be almost useless for intracellular recording. Too high or too low a cell density would either reduce clarity of vision or limit the number of mature neurones present in the culture.

A plating technique was adopted which gave a reasonable density where required and a lower cell density elsewhere, the centre-spot plating method. With this technique, the highest density of cells was in the centre of the culture dish. The density gradually decreased towards the edge of the dish. These cultures proved to be perfect for intracellular recording because an area which contained

cells with the correct characteristics: e.g. size, morphology and distribution, could be found quite close to the centre of the dish. The advantages of working in the centre of the culture dish were the greater space for manoeuvring the electrodes and the better phase contrast optics. In areas of comparable densities, no differences were seen in the relative proportions or the morphologies of the cells present between the two techniques. Therefore, the centre-spot method was adopted. This method also reduced the wastage of cells by increasing the number of culture dishes produced by each dissection.

E] The usefulness of a cell culture was determined by the density of cells on it. At higher densities [$>8.5 \times 10^3$ viable cells per cm^2], the fibroblast layer was not strong enough or sufficiently well formed to hold such a large number of cells. These cultures became confluent after ≤ 48 hours in vitro. After two weeks in culture whole patches of cells would either break away from the main cell layer or, be pulled into a large clump. This effect was especially noticeable around the edge of the culture dish where the highest densities were encountered. The pH of the culture medium [as measured by phenol red dye] became acid within 18 hours of a medium change. Relative numbers of neurones in these cultures also appeared to decline more rapidly than normal. After three weeks in vitro observations became increasingly difficult because of the overgrowth, with cells apparently forming 2 or 3 layers. Neurones were often present in large clumps. These were impossible to work with because of their lack of isolation and the reduced optical quality [cf. fig II.6b].

At lower densities [$<3 \times 10^3$ viable cells per cm^2] the cells took longer than 1 week in vitro to become confluent. After 2 weeks in vitro and therefore after treatment with FuDR, the cell layer was again intermittent [fig II.6c]. The majority of surviving neurones had small perikarya and extremely long processes when compared with those from normal density cultures [cf. fig II.5c and fig II.6c].

At the optimum density, [between 4 and 8×10^3 viable cells per cm^2 of culture dish] the cells became confluent between 3 and 4 days in vitro. Neurones were visible both singly and in clusters/groups.

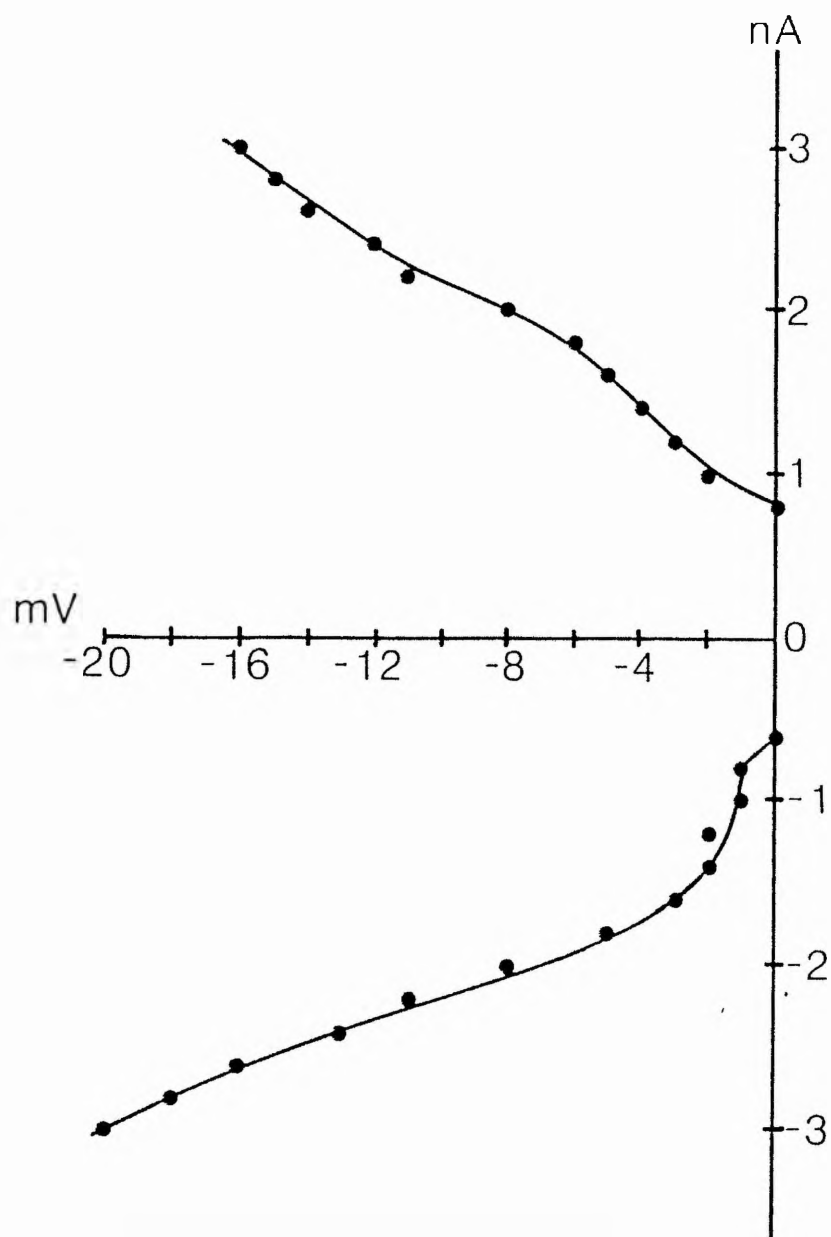
These modifications: a] reduced time spent on the dissection, b] possibly increased the heterogeneity of the cells in the final culture, c] reduced the chance of the culture becoming contaminated during preparation and, d] increased the number of culture dishes produced for each dissection.

FIGURE IV.1

CURRENT/VOLTAGE RELATIONSHIP [RECORDING ELECTRODE]

A current [nA]/voltage [mV] relationship for a 70Megaohm resistance recording electrode, tipette; similar effects were also seen with the standard type of recording electrode. This graph indicates the problem with electrodes of either tipette or standard design. When currents in excess of 1nA were passed through the electrode an appreciable change in the electrode resistance occurred, however, this would not be as obvious when recording from a neurone.

The electrolyte was 2M potassium acetate and the electrode was immersed in normal recording medium.



IV.2] CELL MORPHOLOGY AND ELECTROPHYSIOLOGY IN CULTURE

A wide variety of cell types were present in the cultures. The following section describes the individual and group morphologies of the different cell types and electrophysiological characteristics of glia, cardiac myocytes and neurones.

The following electrophysiological data were obtained using a single electrode current clamp unit. The nature of this study made the use of electrodes with high resistances [between 40 and 150 Megaohms] a necessity. The following variety of electrical artefacts were usually introduced by these electrodes. Rectification by the electrode could be very similar to that seen/expected from the membrane. To overcome this, the only electrodes used were those which showed no rectification when between $\pm 0.6\text{nA}$ of current was flowing through them in recording medium. Some of the better electrodes allowed up to $\pm 1.2\text{nA}$ to pass without any significant rectification. To illustrate this problem, the graph in fig IV.1 shows the current/voltage relationship for an electrode of 70 Megaohms resistance. The resistance of the electrode could also limit the current passing ability. A capacity compensation was built into the preamplifier which was capable of dealing with any reasonable electrode capacitance and therefore electrode capacitance was not so much of a problem. Great care was taken to keep within the limits of the electrode when changing membrane potential of the cell or constructing current/voltage relationships.

IV.2.1] SMOOTH MUSCLE

In co-cultures of ileum and spinal cord, a large proportion of the non-neuronal cell types appeared to be smooth muscle. They averaged 5um to 10um in width and appeared up to 100um long. No distinct features were visible except for a nucleus and the tapered shape. Occasionally, spontaneous contractions were observed.

The effect of having a large number of smooth muscle cells in the cell layer was devastating. After 3 weeks in vitro these cells had formed bundles [fibres] which were capable of pulling large areas of the cell layer away from the walls of the culture dish. In some cases this exposed over 50% of the base of the culture dish.

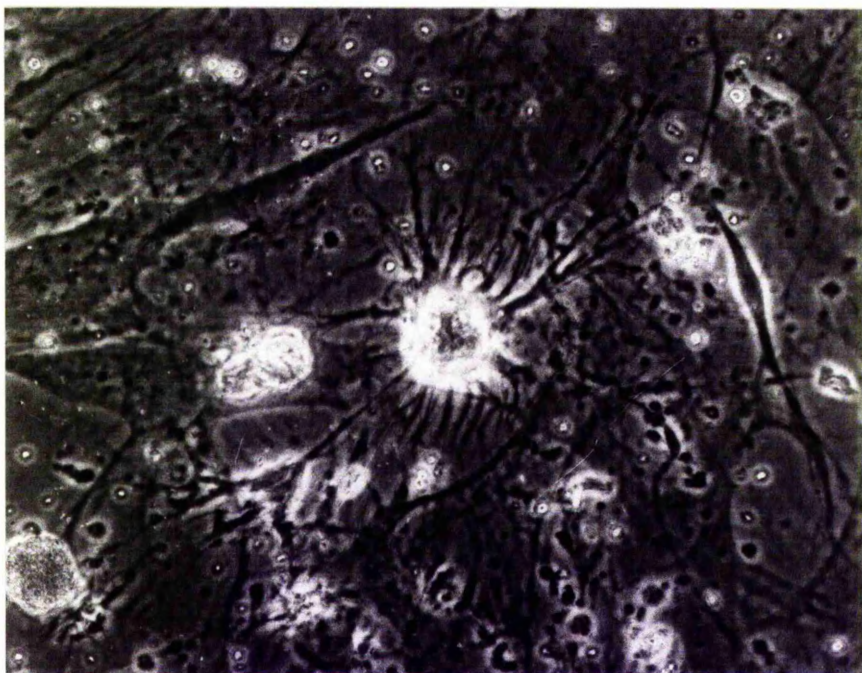
IV.2.2] SKELETAL MUSCLE

These cells were seen on very few occasions. An attempt was made to grow them by co-culturing parts of the embryo expected to contain this tissue [e.g. hind limb]. Results from these attempts were inconclusive as so few skeletal muscle cells were seen. Striations were faintly visible in the muscles when viewed under phase contrast illumination. These cells were approximately 20um in diameter and appeared up to 200um long. Spontaneous contractions with no obvious rhythm were associated with these cells. Skeletal muscle cells were found in bundles which would occasionally cross. However, there was apparently no electrical coupling; i.e., the contractions did not cross from one bundle to another.

a



b



c

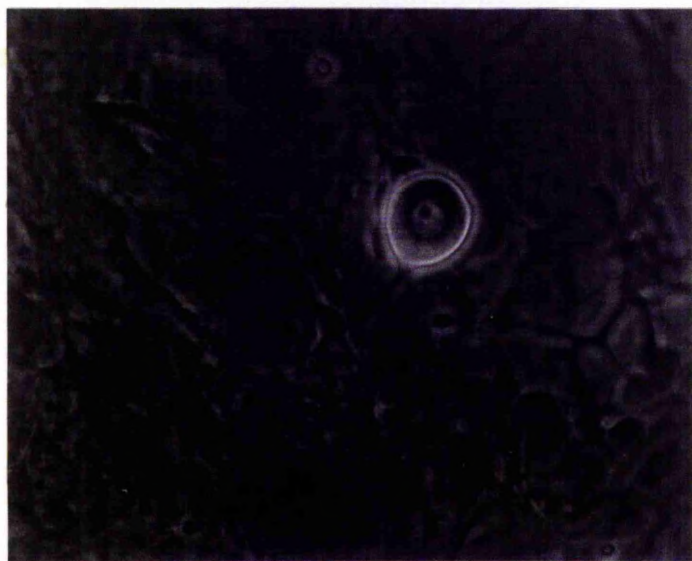


FIGURE IV.2

CELL TYPES FOUND IN THE CULTURES.

a] Epithelial cells, including a blister formation.

Scale bar 40um.

b] Glial cell, note the multitude of processes.

Scale bar 40um.

c] Dorsal root ganglion neurone, with the single process and "halo". Also note the distinct nucleolus.

Scale bar 30um.

Phase contrast illumination was used to view the cells.

FIGURE IV.3

ACTION POTENTIAL FIRING PATTERNS

Intracellular voltage recordings from a cardiac myocyte [top], a spinal neurone [middle] and a dorsal root ganglion neurone [bottom], made in normal recording medium. The action potential recordings were attenuated by the pen recorder.

A] CARDIAC MYOCYTE

Spontaneous activity of the cell. Between bursts of action potentials the membrane potential was relatively static, remaining at resting levels [-60mV]. Scale bars 20mV and 5sec [left] or 200msec [right].

B] CULTURED NEURONES

The solid bar underlying both voltage recordings denotes the injection of current into the neurone. The lower level was 0nA. Scale bars for neurone recordings: 10mV or 1nA vertical bar, 1s horizontal bar.

I] SPINAL NEURONE

On injection of current there was a burst of action potentials. The rate of firing decreased slightly with time. Resting membrane potential -54mV.

II] DORSAL ROOT GANGLION NEURONE

Injection of positive current produced a depolarization which evoked a single action potential. This action potential was followed by a large after-hyperpolarization. This activity was typical of most, if not all of these neurones. Resting membrane potential -56mV.

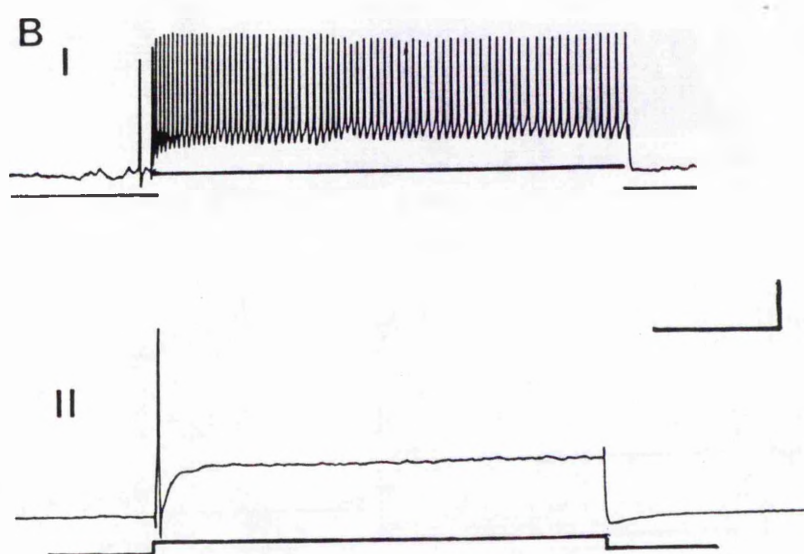
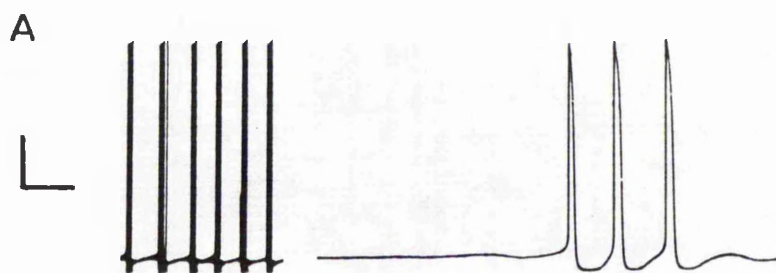


FIGURE IV.4

MEMBRANE ACTION POTENTIALS

Voltage recordings from a cardiac myocyte [top], a dorsal root ganglion neurone [middle] and a spinal neurone [bottom]. Photographed or drawn directly from the oscilloscope screen, using a Polaroid camera. Comparison of action potential parameters; the muscle action potential was part of spontaneous activity as described in fig IV.3; the neuronal action potentials were evoked on the off phase of a constant current hyperpolarizing pulse [200msec duration, -0.5nA].

A] Cardiac myocyte action potential, scale bars 50mV and 50msec.

Resting membrane potential -70mV .

B] DRG action potential, scale bars 20mV and 1msec.

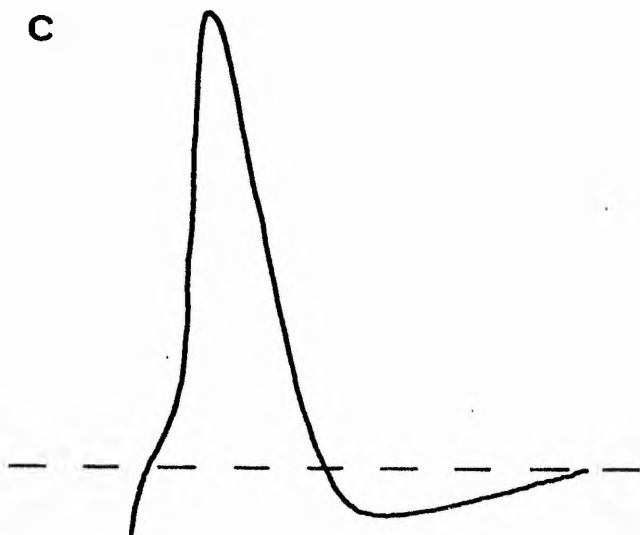
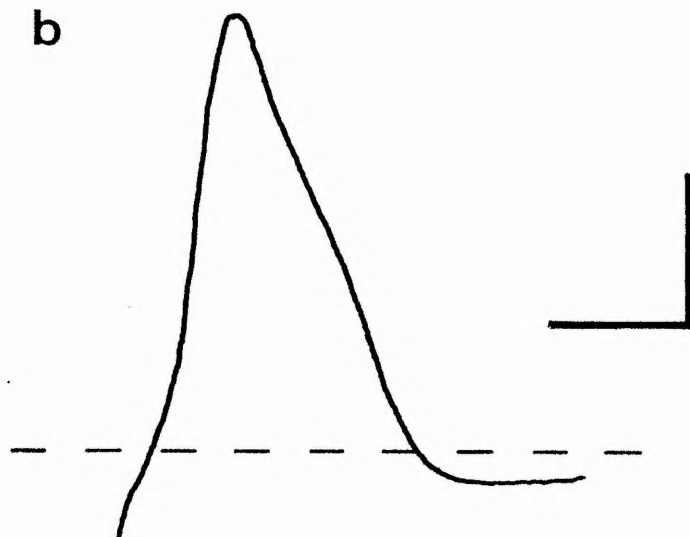
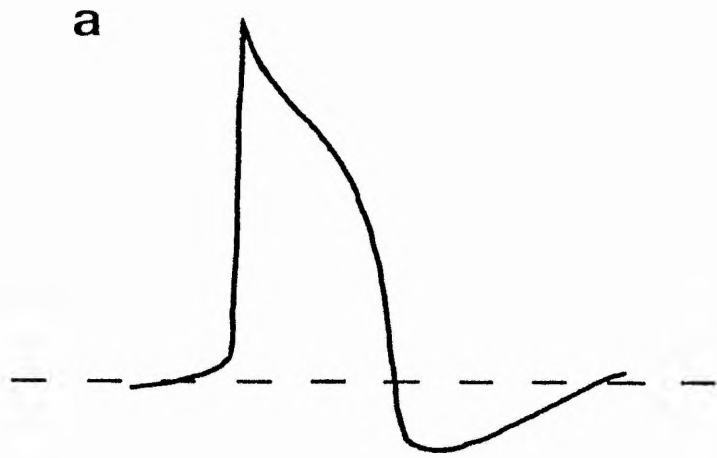
Resting membrane potential -50mV .

C] Spinal neurone action potential, scale bars 20mV and 1msec.

Resting membrane potential -56mV .

All recordings were made in normal recording medium.

Resting membrane potentials denoted by the dotted line.



IV.2.3] CARDIAC MUSCLE

Mixtures of cardiac tissue and spinal cords from the same embryo were successfully co-cultured. Possibly two types of muscle cell were seen. One type was small and always found in clumps, this made any accurate morphological description difficult. They were approximately 10um in diameter and appeared to have webbed processes. These cells appeared similar to rat myocytes in culture [Mark and Strasser, 1966]. The other cell type was found both isolated and in clumps. These were approximately 10um wide and up to 100um long, occasionally striations were seen. These appeared very similar to "well differentiated" rat myofibrils in culture [Mark and Strasser, 1966]. The striations were more obvious in the fibre-like bundles of these cells. Both cell types appeared to be on top of the fibroblast layer. Furthermore, processes from neurones were seen to pass through the clumps and bundles of the muscle.

All of these muscle cells [units] exhibited synchronous, temperature dependent, rhythmical contractions [beating]. The contractions were seen in both culture media and recording media. Beating ceased after replacing the culture medium with normal recording medium at room temperature. Similar effects were seen after other rapid drops in temperature; e.g. after placing the dish into a refrigerator. After a period of 5 to 10 min the beating resumed, albeit at a lower rate.

Recordings were made from ten rhythmically beating muscle cells, apparently of cardiac origin. The electrolyte used in the recording

electrodes was 2M potassium acetate and the cells were bathed in normal recording medium at room temperature [between 18 and 23°C]. These cells had resting membrane potentials of between -70 and -80mV. This range is comparable to that found by Sperelakis [1972] for chick embryo myocytes [-40 to -90mV] and Taniguchi, Kokubun, Noma and Irisawa, [1981] for rabbit ventricular myocytes [-70.3 \pm 6mV]. Action potentials were generated spontaneously by these cells. The action potentials overshoot the zero potential by between 20 and 30mV and had a duration of 50msec, fig IV.3a. The activity was in bursts, each consisting of a series of action potentials, usually 3 or 4. The burst of action potentials was synchronous with a mild contraction of the muscle cell. There were no membrane potential changes between the bursts, fig IV.4a. This type of activity appeared to be following a pacemaker however, no recordings were made from cells which showed the expected pacemaker activity. Input resistance measurements were hampered by the spontaneous firing of action potentials and the subsequent contractions. However, those which were measured were in the range 20 to 50 Megaohms. These values are slightly higher than those measured in cultured chick embryo myocytes [between 5 and 20 Megaohms; Sperelakis, 1972].

IV.2.4] EPITHELIAL CELLS

These were found predominantly in cultures containing tissue from hind limb or ileum, but were also found in most other cultures. Their shape was typical of cultured epithelia, [cf. confluent MDCK cells, with fig IV.2a]. They appeared to form monolayers, the shape of an individual cell being determined by the number of neighbouring

cells. Usually the cells were 10 to 20um in diameter, the depth was not accurately measured but appeared to be between 1 and 5um. No other cells were seen to grow over or under the epithelial sheet. However, the presence of a fibroblast monolayer underneath cannot be ruled out without a further study. Occasionally a raised area, similar to a blister, formed in the approximate centre of the monolayer, fig IV.2a. Apparently, this was also only one cell thick.

IV.2.5] GLIAL CELLS

The neuroglia were present, although not obviously so, in all cultures containing CNS tissue. In many cases, their appearance was similar to that of neurones, a roughly spherical cell body with numerous processes coming from it, [cf. Ransom et al., 1977a with fig IV.4b]. The cell bodies were between 10 and 50um in diameter.

Lucifer yellow dye injection was successfully performed into four of these cells. Two of these showed dye coupling: e.g. other cells, which had not been impaled also contained sufficient dye to fluoresce [Stewart, 1981]. The fluorescence of the coupled cells was not as intense as that of the impaled cell, fig IV.5. The coupled cell may, or may not have been a glial cell [Viancour, Bittner and Ballinger, 1981].

FIGURE IV.5

DYE INJECTION [GLIAL CELL]

The photomicrograph shows a cell which has been filled with Lucifer yellow CH. The cell was viewed with ultraviolet illumination.

The drawing is a representation of this cell constructed from photographic negatives. This cell [arrow] was electrically a glial cell. Dye coupled cells can also be seen. Resting membrane potential -38mV . Injection parameters: constant injection of -0.2nA for 10 min.

Scale bar $70\mu\text{m}$

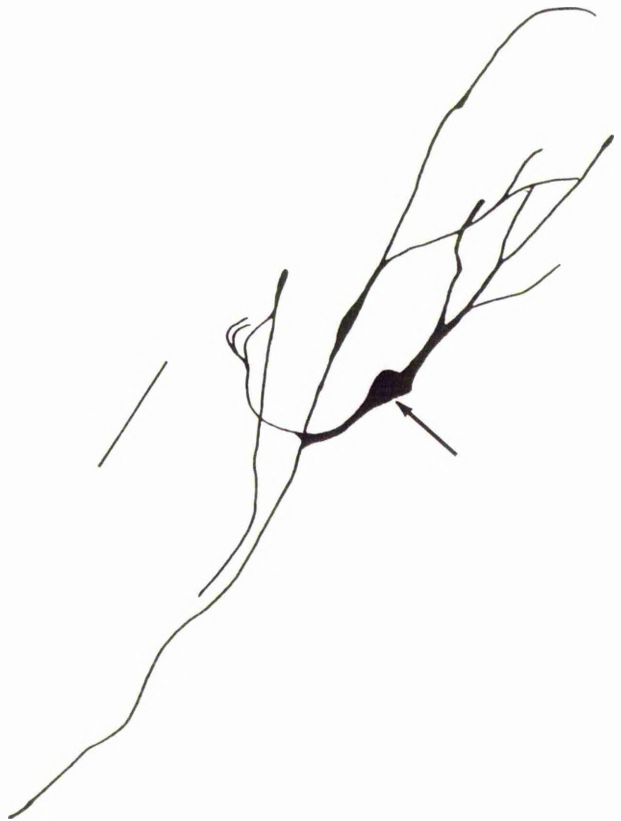


FIGURE IV.6

DYE INJECTION

Representation of a glial cell after Lucifer yellow CH dye injection. The cell was viewed with ultraviolet illumination.

Scale bar 40 μ m

Resting membrane potential -60mV.

Injection parameters: pulsatile injection of -0.5nA [200msec duration at 1Hz] for 15 min.

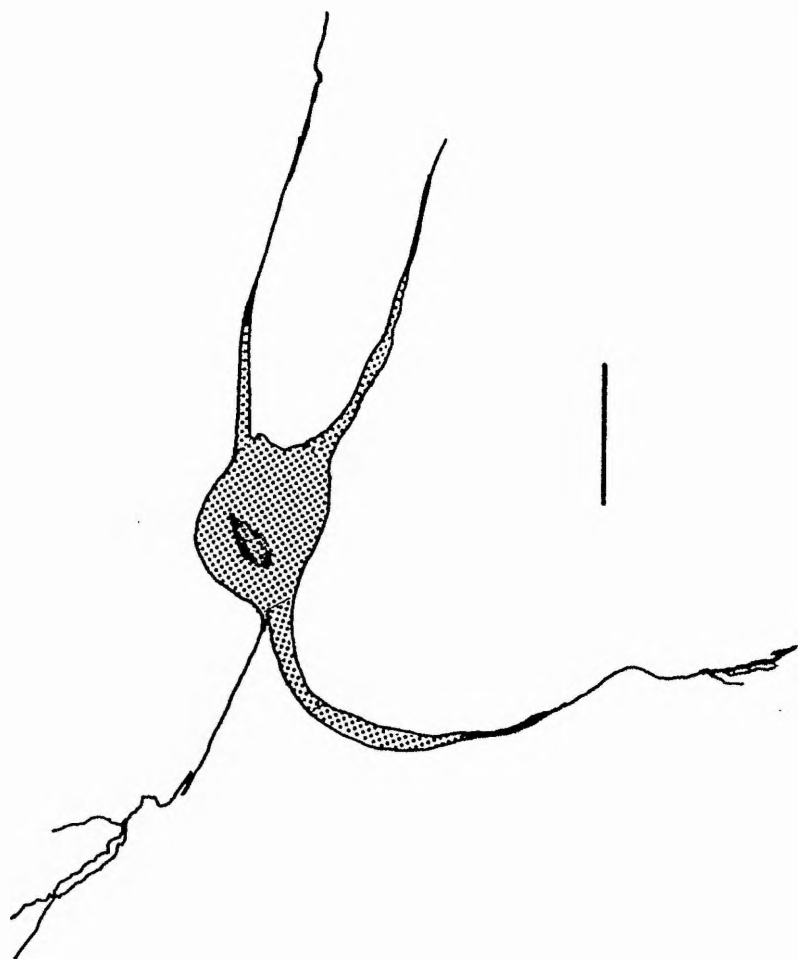


FIGURE IV.7

CURRENT/VOLTAGE RELATIONSHIP [GLIAL CELL]

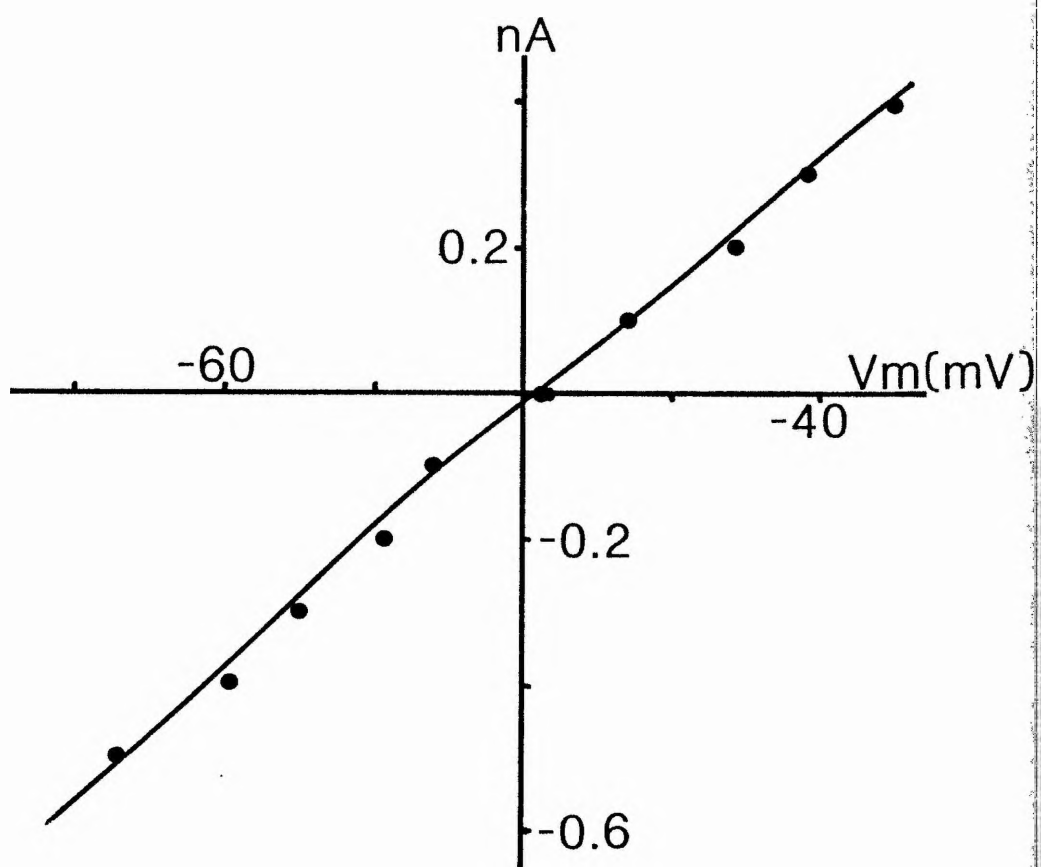
Current [nA]/Voltage [mV] relationship for a glial cell.

Note the apparent lack of any rectification.

Current steps of ± 0.1 nA were used.

Resting membrane potential -48mV.

Input resistance of the cell 27 Megaohm.



Intracellular recordings were made from 11 cells, using electrodes which contained 2M potassium acetate as the electrolyte. The average resting membrane potential was -47.4 ± 9.4 mV [n=11]. This compares favourably with the measurements of Ransom *et al.*, [1977a]. Input resistance measurements were made from six of these cells, the average was 20.8 ± 4.3 Megaohms [n=6]. These results also compare favourably with those of Ransom and co-workers [1977a]. However, the results from both of these studies [Ransom *et al.*, 1977a and this thesis] are greater than those reported by Kettenman, Sonnhof, Camerer, Kuhlmann, Orkland and Schachner [1984]. Kettenman and co-workers made recordings from a sub-group of neuro-glia [oligodendrocytes] and found resting membrane potentials of circa -70mV and input resistances of circa 3.3 Megaohms [from 0.7 to 16 Megaohms]. The results from this group suggest that the glial cells studied in this thesis and by Ransom *et al.*, [1977a] were not exclusively oligodendrocytes. No action potentials or other activity were seen in any of these cells. The current/voltage relationships showed little or no signs of delayed and anomalous rectification, fig IV.7, which is very similar to results reported by Ransom and co-workers, [1977a].

FIGURE IV.8

DYE INJECTION [DORSAL ROOT GANGLION NEURONE]

Upper: photomicrograph of a DRG neurone containing Lucifer yellow CH-fluorescence.

Lower: representation of the above neurone made from photographic negatives.

Scale bar 100 μ m.

Resting membrane potential -55mV.

Injection parameters: constant injection of -0.2nA for 20 min.

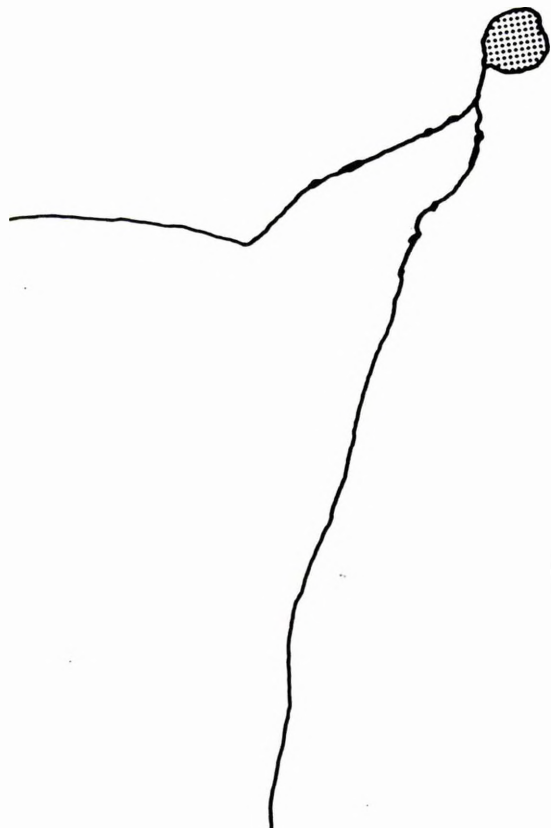
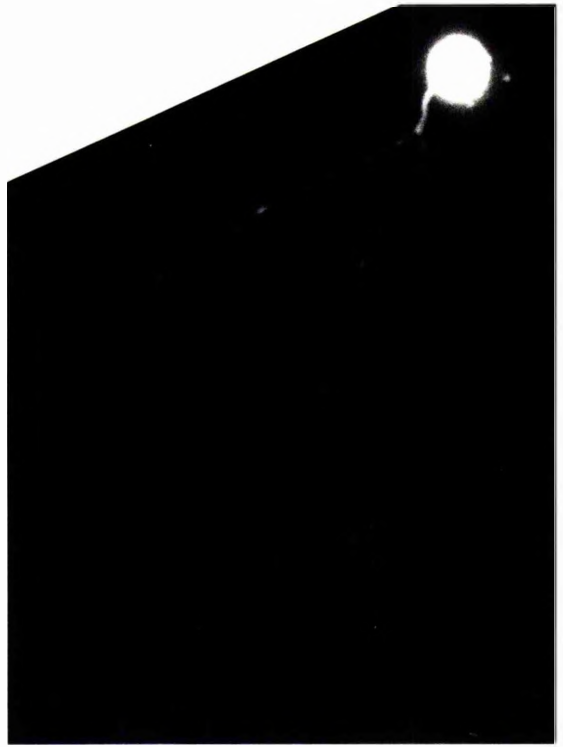


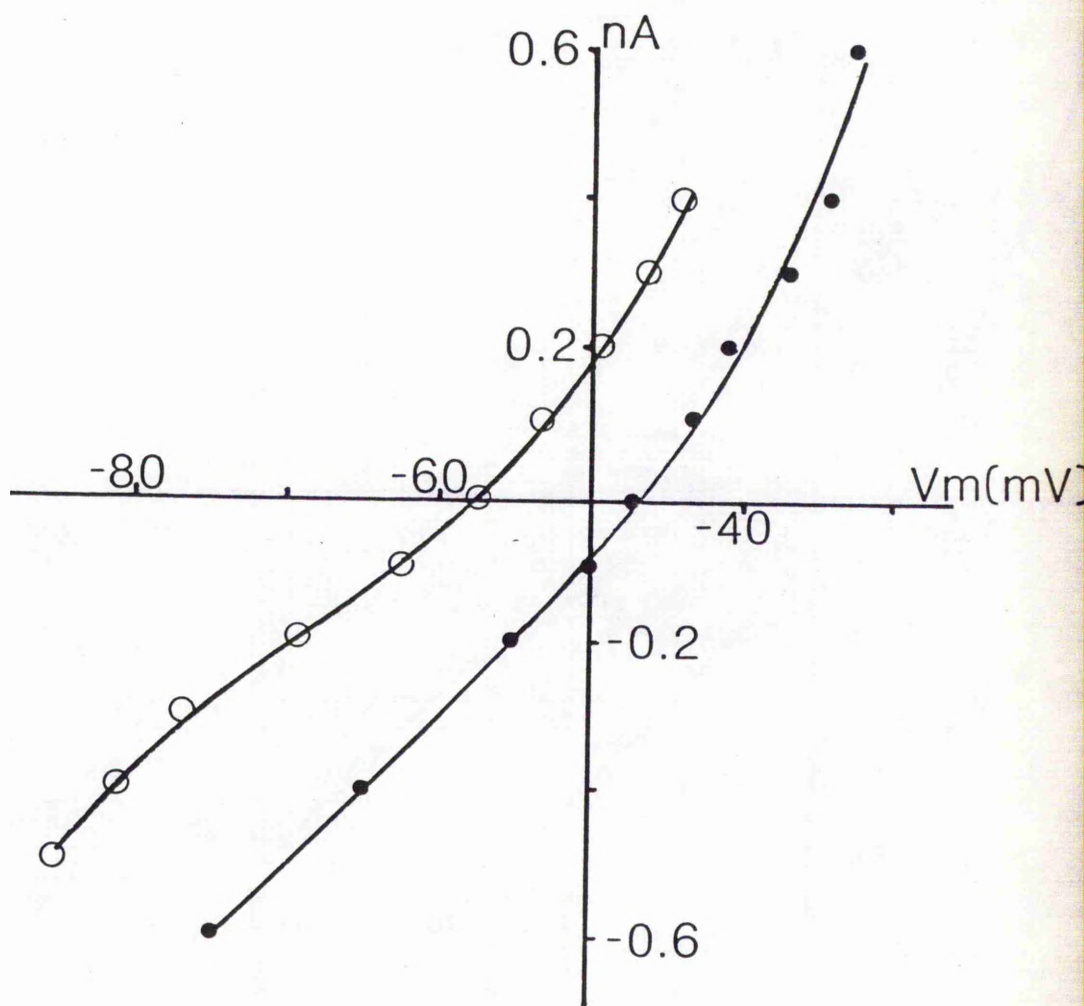
FIGURE IV.9

CURRENT/VOLTAGE RELATIONSHIP [DRG NEURONES]

Current [nA]/voltage [mV] relationships for two DRG neurones. Both delayed and anomalous rectification were apparent, albeit not obviously so.

Current steps of ± 0.1 or ± 0.2 nA were used.

Resting membrane potentials were -48 mV [●] and -58 mV [○].



IV.2.6] DORSAL ROOT GANGLION NEURONES

These were a morphologically distinct group of neurones found in all the cultures which contained spinal cord cells, [cf. Peacock, Nelson, and Goldstone, 1973; Ransom *et al.*, 1977a]. They were distinctive because of their apparent lack of processes and the appearance of the perikaryon under phase contrast illumination. Figure IV.2c shows a typical DRG neurone which had been *in vitro* for 4 weeks. The perikaryon was almost spherical and, when viewed with phase contrast optics, was usually surrounded by a halo of light. The nucleus usually contained a distinct nucleolus. Perikarya were between 10 and 50um in diameter. On careful examination a single process could be seen. This process was usually quite thin and consequently could not be followed for any great distance, under phase contrast illumination. Approximately 10% of the DRG neuronal perikarya were apparently not attached to the culture dish, other than by their process. A small percentage of the DRG neurones were flat and had 2 or 3 processes, especially in younger cultures [up to 3.5 weeks *in vitro*]. These cells were distinguished by their electrophysiological characteristics alone.

Lucifer yellow CH was successfully injected into seven of these neurones, which had been 3 to 6 weeks *in vitro*. The dye revealed that only one process left the perikaryon. This process bifurcated at some point along its length, fig IV.8. Low numbers of processes were also reported by Peacock *et al.*, [1973] and Ransom *et al.*, [1977a]. On 2 occasions the 2 resulting processes also bifurcated [this can also be seen in fig 2e of Mudge, 1984]. However, the

fluorescence was too low to allow photography of this phenomenon.

Apart from being morphologically distinct, these were also an electrophysiologically separate group of neurones. Recordings were made using electrodes filled with 2M potassium acetate. The average resting potential was -57.9 ± 6.9 mV [$n=45$] with an average input resistance of 35.0 ± 5.0 Megaohms [$n=19$]. These results compare favourably with those of Peacock *et al.*, [1973], Ransom *et al.*, [1977a] and Heyer and MacDonald [1982]. Spontaneous e.p.s.p.s and i.p.s.p.s were not seen. Spontaneous action potentials were occasionally found although these were usually associated with injury.

Action potentials could be evoked by injecting pulses of positive current into the perikaryon. The average amplitude of these action potentials was 72mV [$n=19$], with an average overshoot [of the zero potential] of 14.1 ± 4.0 mV [$n=19$] and an average duration of 4.1 ± 5.0 msec [$n=19$], measured at the base of the action potential. The action potential had a distinctive shape [fig IV.4b], with a rise time of between 70 and 90V/sec. This range of values is slightly lower than those reported by Ransom *et al.*, [1977a]. The decay phase appeared to have three stages: a] a rapid decay from the peak value to approximately 0mV at a rate of 60 to 80V/sec, b] a decrease in rate of decay to between 50 and 60V/sec and, c] a return towards the more rapid rate of decay signalled onset of the last stage and the subsequent termination of the action potential. The slowing-down in decay of the action potential [the second part of the repolarization] was possibly caused by the activation of a calcium conductance [Heyer

and MacDonald, 1982]. An after-hyperpolarization was seen following the action potential when the cell was at resting membrane potential.

Prolonged positive pulses only evoked a single action potential from DRG neurones, fig IV.3b. Repetitive short positive pulses would evoke a single action potential each but only over short periods. Eventually, no amount of positive current injection could evoke an action potential from the neurone. After leaving the neurone at resting potential for up to 20 min., action potentials could again be evoked on current injection. However, the ability of the neurone to fire action potentials would again be short term.

Current/voltage relationships showed the presence of both anomalous and delayed rectification in these neurones, fig IV.9. The onset of each of these was circa -80 and circa -45mV.

IV.2.7] OTHER [SPINAL] NEURONES

These neurones were found in all cultures which were derived from the spinal cord. Perikaryon dimensions were from 10 to 60um in diameter and less than 10um in depth. It was possible that neurones were smaller than this however, those neurones were not tested in this study [however, cf. Jackson, Lecar, Brenneman, Fitzgerald and Nelson, 1982]. These neurones usually had more than one process issuing from the perikaryon.

The perikarya of spinal neurones were found both singly as well as in clusters. In the large clusters of cells, it was difficult to record and apply the drugs discretely to a single neurone. The processes and even some of the smaller cell bodies were difficult to resolve because of the close packed nature of these areas. In such areas the major advantages of the culture were negated and therefore those cells were seldom impaled. Partially isolated neurones [figs II.5c and II.6a] were used mainly because of the increased control over the site of drug administration. However, checks were made to ensure that peptide responses, if any, from the neurones in those clusters were not being missed. Occasionally, neuronal processes formed large bundles, most often seen sprouting from the large clusters. These bundles of processes were not necessarily attached to the underlying monolayer and could extend for over 300um. Perikarya were sometimes seen lying in or around these bundles.

' There appeared to be a wide range of neuronal types in these cultures.

FIGURE IV.10

DYE INJECTION [SPINAL NEURONE]

Multipolar neurone after dye injection with Lucifer yellow CH. Viewed with ultraviolet illumination. The figure is a representation drawn from the photographic negatives. One process [arrows] extended circa twice as far as the others, [at least 400 μ m].

Scale bar 50 μ m

Resting membrane potential -58mV.

Injection parameters: pulsatile injection of -0.5nA [200msec duration at 1Hz] for 20 min.

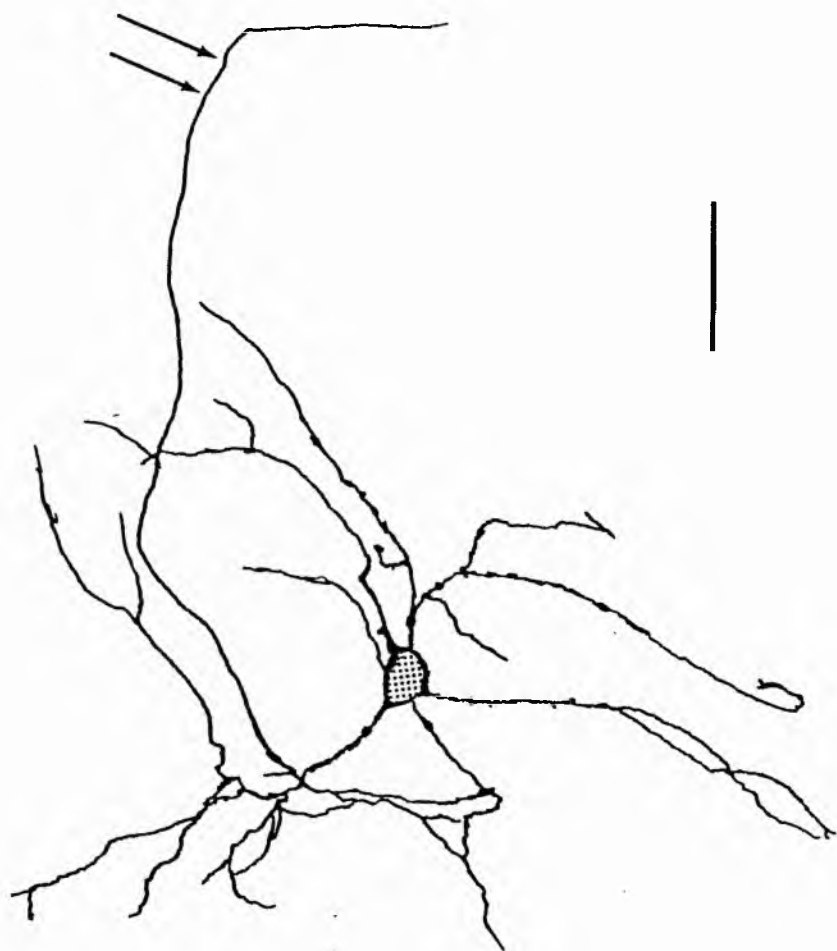


FIGURE IV.11

DYE INJECTION [SPINAL NEURONE]

Lucifer yellow CH dye injected neurone, viewed with ultraviolet illumination. The figure shows a representation of a tripolar spinal neurone drawn from the photographic negatives.

Scale bar 50 μ m.

Resting membrane potential -48mV.

Injection parameters: constant injection of -0.2nA for 10 min.



FIGURE IV.12

DYE INJECTION [SPINAL NEURONE]

Fluorescence photomicrograph and representation of a
Lucifer yellow CH filled spinal neurone.

Scale bar 50 μ m.

Resting membrane potential -54mV.

Injection parameters: pulsatile injection of -0.5nA [200msec
duration at 1Hz] for 22 min.

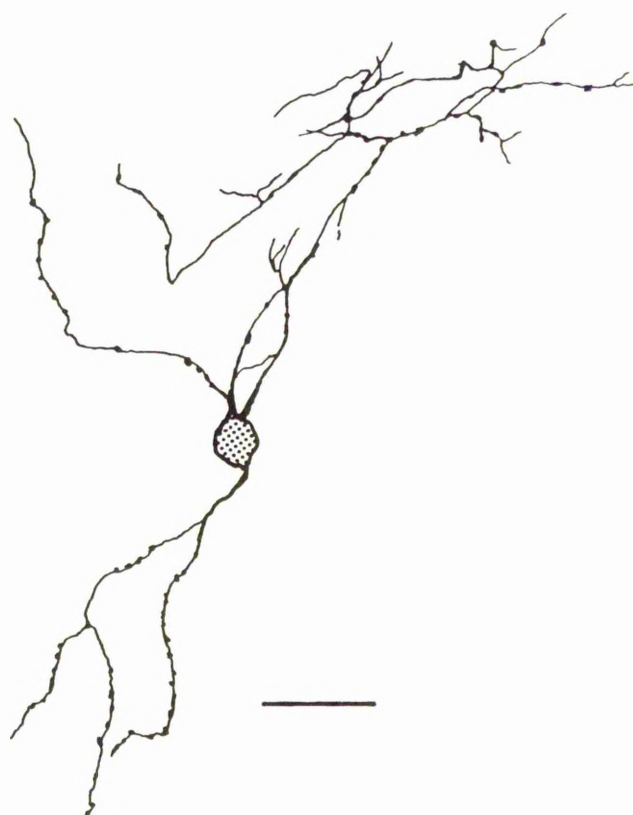
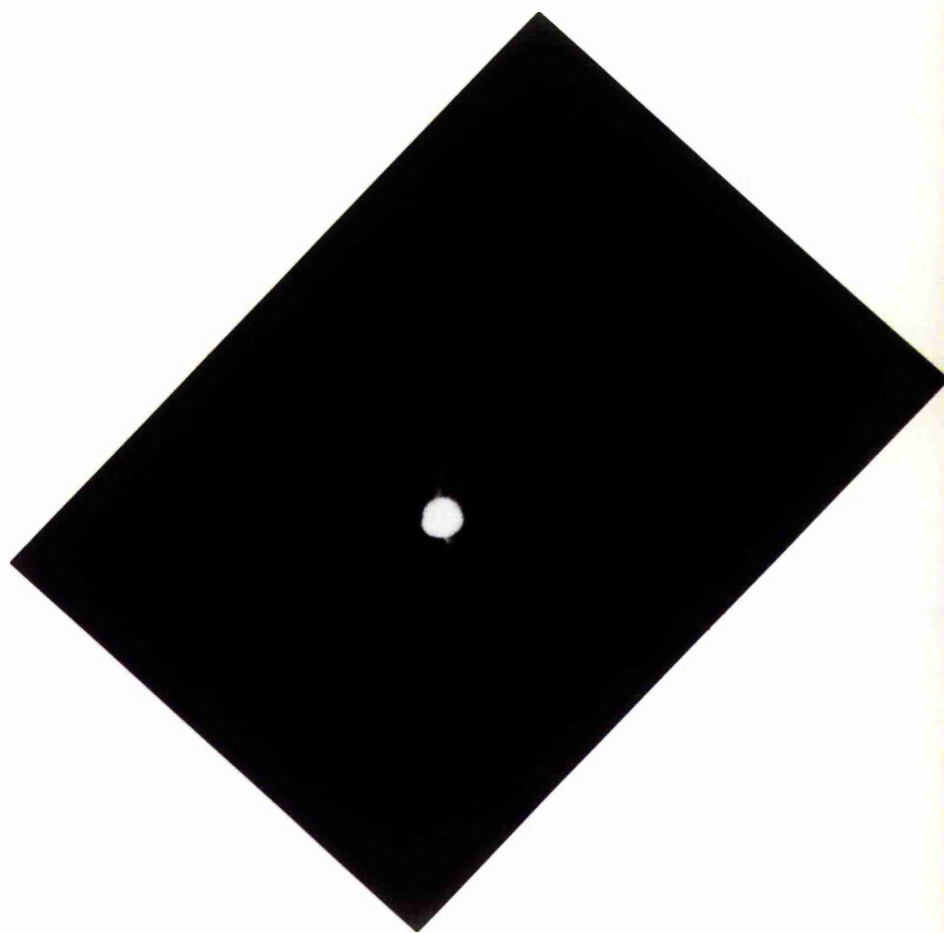


FIGURE IV.13

DYE INJECTION AND APPARENT DYE COUPLING [SPINAL NEURONE]

Photomicrograph and graphical representation of a spinal neurone after Lucifer yellow CH dye injection: note the adjacent cell which was also filled with dye. The cell with the less intense fluorescence was not directly dye injected. Viewed with ultraviolet illumination.

Scale bar 60 μ m.

Resting membrane potential of the injected cell -58mV.

Injection parameters: constant injection of -0.2nA for 25 min.



In an attempt to classify some of the neurones a series of Lucifer yellow CH dye injections were made. Of the 30 successful dye injections, at least three types of morphology were distinguished, figs IV.10,11 and 12. On two occasions dye coupling was seen between the impaled neurone and another neurone/cell; one of these is shown in fig IV.13 [cf. Stewart, 1981; Viancour et al. 1981]. Each of the dye coupled cells had the appearance of neurones, however, it is possible that either or both were glial cells. It was found from these studies that any distinction between cell type made under the phase contrast illumination was unreliable.

These cells were recorded from using electrodes filled with 2M potassium acetate or 2M potassium chloride. The average resting membrane potential was -56.1 ± 6.6 mV [n=803] which was slightly more negative than reported by Peacock et al., [1973], Ransom et al., [1977a] and Heyer and MacDonald [1982]. However, the results of studies with intact tissue have shown the resting potentials to be even more negative [Murase and Randic, 1983; Stanzione and Zieglansberger, 1983]. This effect was possibly caused by the lack of a complete glial "coat" around the neurones in culture. This would allow the levels of potassium on the external surface of the neurone to become higher, thus making the resting potential more positive [Hertz and Chabin, 1982]. However, neurones were not used if, after being impaled for 15 min, their membrane potentials were more positive than -40mV. The most negative resting membrane potential was -76mV. The average input resistance was 30.9 ± 8.1 Megaohms [n=104], the highest measured was 85 Megaohms and the lowest acceptable was 5 Megaohms. Neurones recorded from for over 15 min. usually had parameters above the cut-off levels, cells with parameters outside those levels either died or "recovered".

FIGURE IV.14

CURRENT/VOLTAGE RELATIONSHIP [SPINAL NEURONES]

Current/Voltage relationships for two spinal neurones.
Both delayed and anomalous rectification was in evidence.

Current steps of ± 0.1 and ± 0.2 nA were used.

Resting membrane potentials of -55 mV [○] and -60 mV [●].

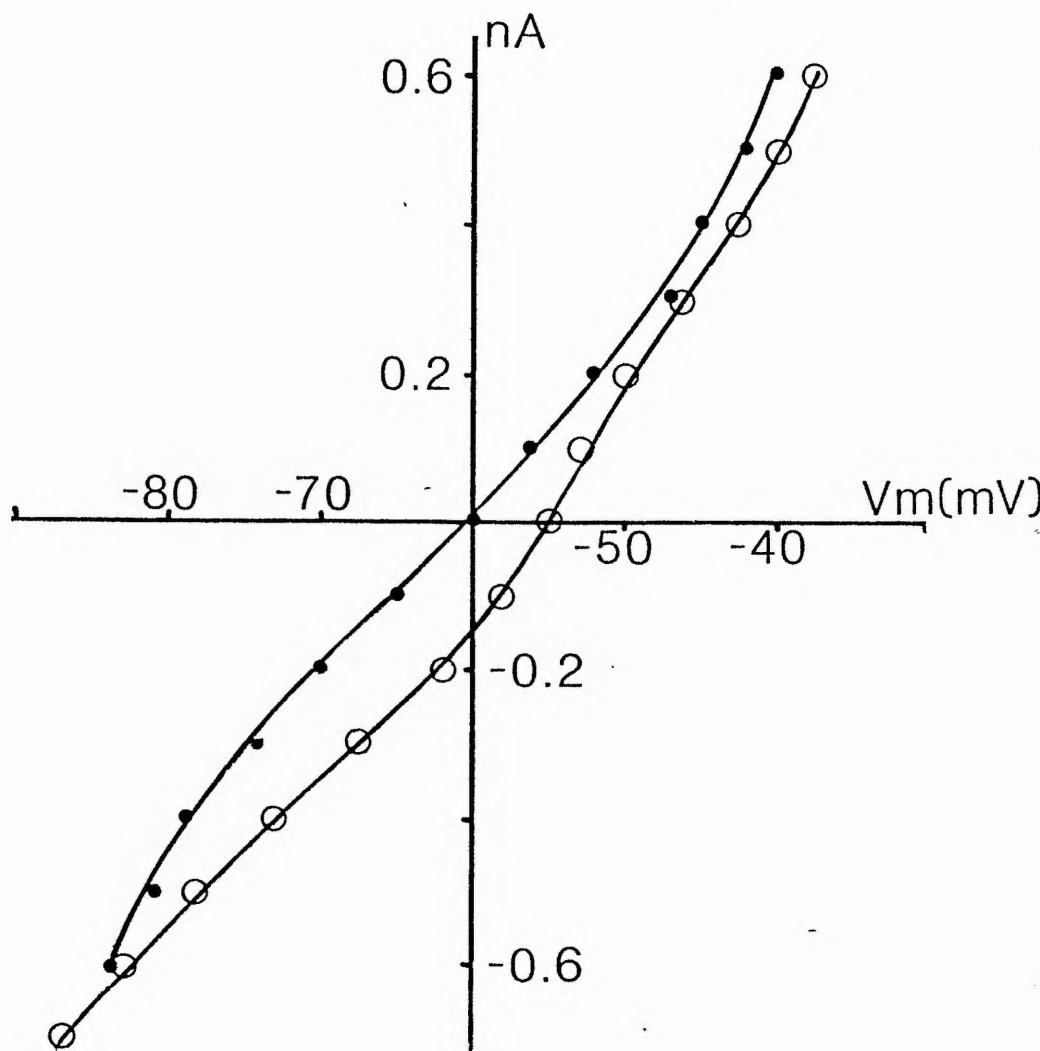


FIGURE IV.15

SPONTANEOUS ELECTRICAL ACTIVITY [FROM SPINAL NEURONES]

Voltage recordings made from spinal neurones. The neurones were bathed in normal recording medium. The spontaneous synaptic activity [i.p.s.p.s and e.p.s.p.s] is shown over a series of membrane potentials.

A] Neurone which had been in culture for 3 weeks. Soma diameter 20um, 2 large processes. Resting membrane potential -50mV. Reversal potential of the activity was circa -50mV.

B] Neurone which had been in culture for 5 weeks. Soma diameter 25um, 3 major processes. Resting membrane potential -45mV. Reversal potentials of the spontaneous activity were circa -30 and -54mV.

Scale bars are featured on the *right* of each series, vertical bar 10mV and horizontal bar 500msec.

A

-30 

-40 

-50 

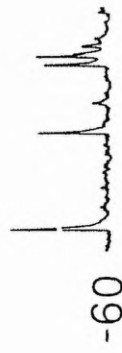
-60 

-90 

b

-35 

-45 

-60 

Action potential parameters were: average amplitude 62mV [n=171], average overshoot 6.2 ± 3.4 mV [n=171] with an average duration of 2.1 ± 1.05 msec [n=171], fig IV.4c. The rise time for action potentials was between 80 and 120V/sec, with decay times only marginally slower, between 50 and 100V/sec. The neurones in this group were capable of long periods of sustained action potential firing; e.g., approximately 300Hz for periods up to 5 min, fig IV.3c. The threshold potential for spontaneous action potential generation was usually between -50 and -40mV. Occasionally, a spinal neurone would produce action potentials which had similar parameters to those produced by DRG neurones. However, these were quite rare; less than 5% of the spinal neurones, [cf. Heyer, MacDonald, Bergey and Nelson, 1981; Heyer and Nelson, 1982].

Current/ voltage relationships for these cells occasionally showed a marked delayed and anomalous rectification, [cf. Ransom et al., 1977a; fig IV.14]. The onset of the rectifiers being more positive than -40 and more negative than -80 ± 5 mV, respectively. This was corroborated in a study of these spinal neurones using a whole cell clamp [Peters, J. personal communication, 1984].

Spontaneous inhibitory and excitatory activity was seen with these cells, fig IV.15. The inhibitory activity had reversal potentials close to the recorded resting membrane potential. On impaling, or re-impaling a cell using potassium chloride as the electrolyte in the recording electrode, the visible activity became excitatory, having a reversal potential of between -20 and -30mV, fig V.8. This inferred a role for chloride ions, which was corroborated by perfusion experiments during which the extracellular chloride ion

concentration was changed.

The excitatory activity had an extrapolated reversal potential of between -15 and 0mV. Apparently, this was unaffected by changing the intracellular chloride ion concentration. This type of activity had the ability to generate action potentials.

In conclusion, the morphology and electrophysiological characteristics of the relevant cells in these cultures appears consistent with those used in other studies. During the period of co-culturing spinal neurones with tissue from other organs no consistent differences were found, with respect to the electrophysiological parameters of the spinal neurones. In addition and more importantly from the point of this project, no consistent increase was seen in the number of neurones which responded to peptide application.

CHAPTER V

RESULTS II

PHARMACOLOGY OF SPINAL NEURONES

V.1] INTRODUCTION

The chemosensitivity of spinal neurones was tested to a variety of amino acids which were known to evoke inhibitory or excitatory responses from vertebrate central neurones in culture. This study allowed a comparison with previously published work on cultured spinal neurones from dissociated mouse spinal cords [Ransom et al., 1977b; Barker and Ransom, 1978; MacDonald and Wojtowicz, 1980; 1982]. Spinal neurones refers to all neurones found in these culture preparations which did not have both the electrophysiological and morphological characteristics of DRG neurones.

The excitatory amino acids used were L-glutamate and analogues, DL-kainate and N-methyl D-aspartate [NMDA]. Those which evoked inhibitory responses from neurones were glycine, gamma-amino butyric acid [GABA]. The GABA analogue, ethylene-diamine [EDA, Perkins and Stone, 1982] was also tested. All of these chemicals were obtained from Sigma Chemicals.

The results from this chapter will be fully discussed in chapter VI.

FIGURE V.1

L-GLUTAMATE RESPONSE

Voltage recordings from a spinal neurone. A permanent record was made using a pen recorder which attenuated fast events [e.g. action potentials, not shown in this figure]. The downward voltage deflections were produced by the injection of constant current pulses, through the recording electrode and into the neurone. Pulse parameters were: duration of 200msec, frequency of 1Hz. The recording electrode contained 2M potassium acetate as the electrolyte. In the "balanced" electrode [section III.7], the voltage deflections gave an indication of the input resistance; a decrease in the size of the voltage deflections would indicate a decrease in the membrane input resistance and vica versa.

L-Glutamate was applied by iontophoresis [20nA, bar].

The response is shown over a range of membrane potentials [V_m] and was recorded in normal recording medium.

Resting membrane potential -57mV

Scale bars 10mV and 2sec.

Vm

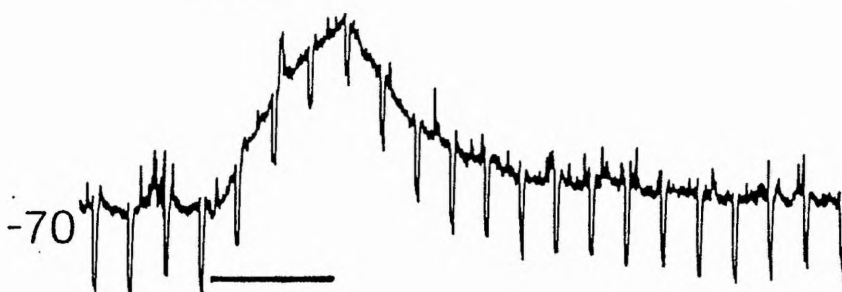
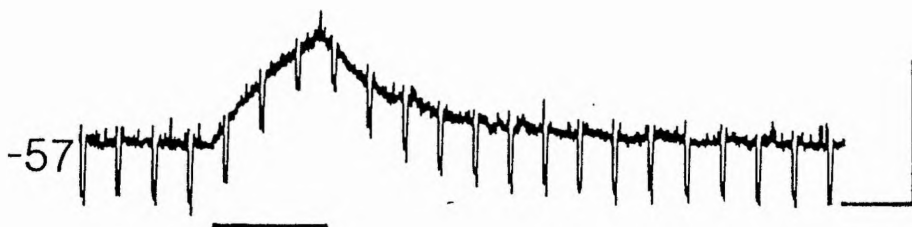


FIGURE V.2

L-GLUTAMATE RESPONSES

Voltage recordings from spinal neurones, details as in fig V.1. L-Glutamate was applied by pressure ejection [50uM, 100msec, arrowheads].

A] A biphasic response showing decrease followed by an increase in membrane resistance; membrane potentials are featured to left of the responses. Recordings were made in high magnesium recording medium. Resting membrane potential -55mV.

B] Response accompanied by a decreased membrane resistance; this was followed by a transient hyperpolarization. Recording made in normal recording medium. Resting membrane potential -50mV.

Scale bars 10mV and 2sec.

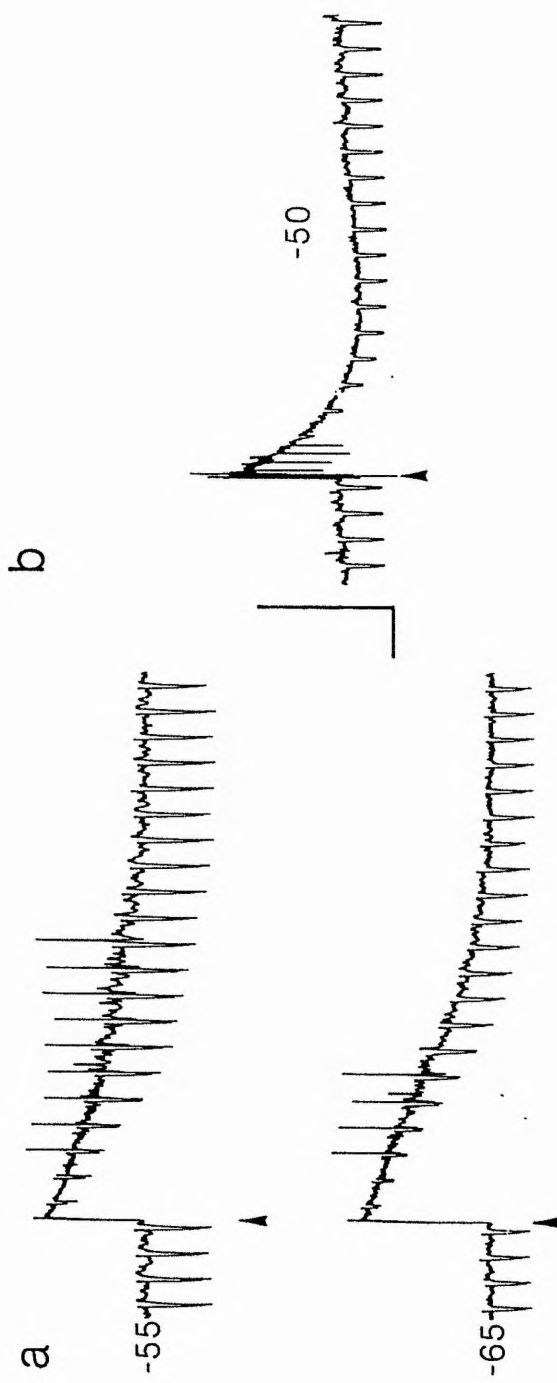


FIGURE V.3

RESPONSE TO DL-KAINATE

Voltage recordings from a spinal neurone, details as in fig V.1. DL-Kainate was applied by pressure ejection [50uM, 50msec, arrowheads]. The response is shown over a range of membrane potentials [Vm].

Recordings were made in normal recording medium.

Resting membrane potential -50mV.

Scale bars 10mV and 2sec.

Vm

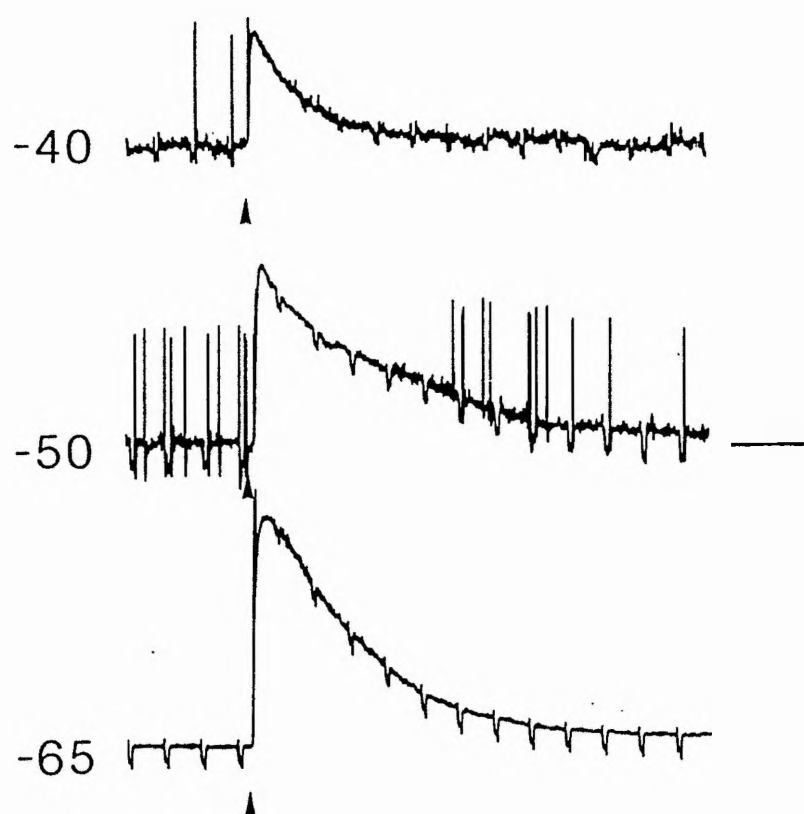


FIGURE V.4

RESPONSE TO NMDA

Voltage recordings from a spinal neurone, details as in fig V.1. NMDA was applied by pressure ejection [50uM, 150msec, arrowheads]. The response can be seen over a range of membrane potentials, [Vm].

Recordings were made in high magnesium recording medium.

Resting membrane potential -60mV

Scale bars 10mV and 2sec.

Vm

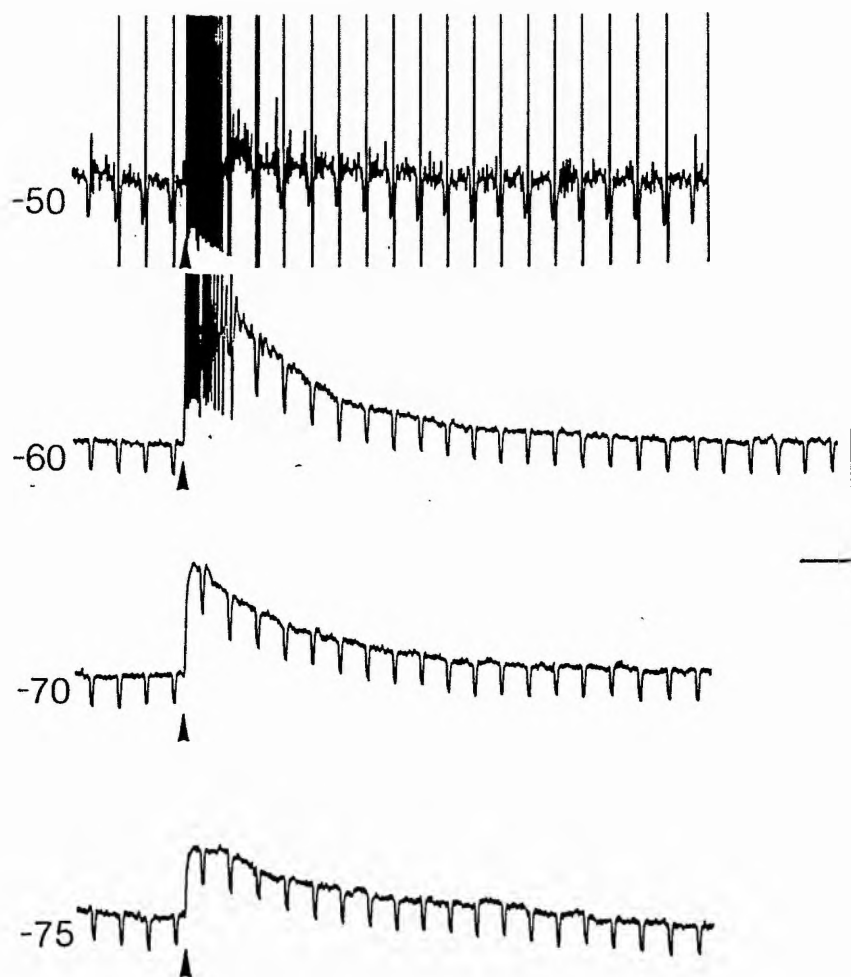


FIGURE V.5

GLYCINE RESPONSE

Voltage recordings made from a spinal neurone, details as for fig V.1. Glycine was applied by pressure ejection [50uM, 200msec, arrowheads]. This response is shown over a range of membrane potentials [Vm], thus, giving an indication of the equilibrium potential, [circa -52mV].

These recordings were made in normal recording medium.

Resting membrane potential -52mV

Scale bars 10mV and 2sec.

Vm

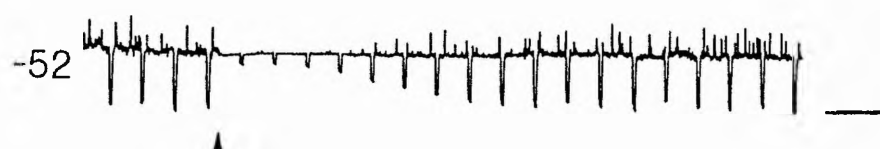


FIGURE V.6.

GLYCINE RESPONSE:

AFFECT OF ALTERING EXTRACELLULAR CHLORIDE CONCENTRATION

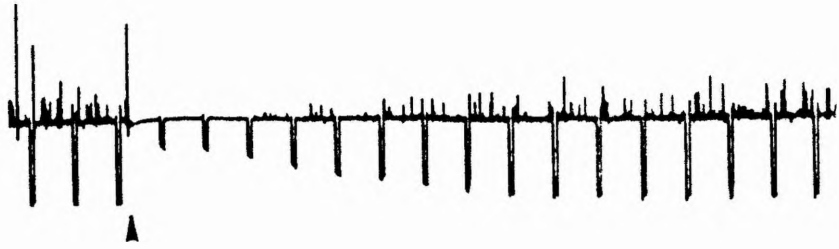
Voltage recording made from a spinal neurone, details as for fig V.1. Glycine was applied by pressure ejection [100uM, 200msec, arrowheads]. The membrane potential in each recording was the same [-56mV].

A] shows the response in normal recording medium [156mM chloride]

B] shows the response in low chloride [75mM] recording medium.

Scale bars 10mV and 2sec

A



B



V.2] EXCITATORY AMINO ACIDS

V.2.1] L-GLUTAMATE

The predominant responses were depolarizations associated with a decrease in input resistance. These had extrapolated reversal potentials of between -5 and -15mVs, fig V.1. Concentrations of between 10 and 100uM L-glutamate [monosodium salt] were used for pressure ejection, whereas the iontophoresis solutions contained 1mM amino acid.

With lower doses of L-glutamate, or in high magnesium medium, a few neurones [c.5% of those tested] responded with a biphasic depolarization. The above response was seen accompanied by a late, small increase in input resistance, fig V.2a. In normal recording medium both responses were accompanied by an increase in synaptic potentials, fig V.2a. A slowly developing and prolonged hyperpolarization followed the L-glutamate evoked depolarization in a further circa 5% of the neurones tested, [fig IV.2b]. This appeared similar to the ouabain-sensitive effects shown by Ransom, Barker and Nelson, [1975].

V.2.2] DL-KAINATE

Each of the 20 spinal neurones tested with DL-kainate responded with a depolarization accompanied by a decreased input resistance. The extrapolated reversal potential was between -5 and -15mVs, fig V.3. An increase in the number of synaptic potentials was sometimes seen, usually the decrease in input resistance abolished the effects

of synaptic input. DL-Kainate was pressure ejected at a concentration of 100uM.

V.2.3] N-METHYL D-ASPARTATE [NMDA]

Fifteen neurones were tested, of which ten gave definite responses. All of those 10 neurones responded with a depolarization accompanied by an increase in input resistance, fig V.4. The equilibrium potential of these responses could be extrapolated to between -85 and -80mV. However, these responses showed a marked voltage dependence in normal recording medium and were not reversible. This made any extrapolation for reversal potential erroneous. During these responses there was an apparent increase in the number of synaptic potentials. Pressure ejection solutions contained NMDA at a concentration of 100uM.

V.3] INHIBITORY AMINO ACIDS

V.3.1] GLYCINE

All of the spinal neurones tested with glycine responded with a hyperpolarization accompanied by a decrease in input resistance. These responses inverted close to the resting membrane potential of the neurone, fig V.5. Application of glycine was by either iontophoresis or pressure ejection, [concentrations were 1mM and 100uM, respectively]. When the extracellular chloride ion concentration was lowered to 75mM [by substitution of sodium chloride for sodium isethionate], the reversal potential of the response became more positive, fig V.6. The reversal potential of these

responses was not affected when the extracellular concentrations of either sodium or potassium were altered.

V.3.2] GAMMA-AMINO BUTYRIC ACID [GABA]

The response to GABA was a hyperpolarization accompanied by a decreased input resistance. This was found in all of the spinal neurones tested. The reversal potential was always close to the resting membrane potential, fig V.7. Application of GABA was made by both iontophoresis and pressure ejection, at concentrations of 1mM and 100uM, respectively.

When neurones were intracellularly loaded with chloride, using potassium chloride as the electrolyte in the recording electrode, the GABA response had a reversal potential which extrapolated to approximately -30mVs, fig V.8. Concurrently, all of the measurable synaptic potentials became *depolarizing*, the majority inverting at a similar potential to the GABA response. On 5 occasions, the above data was corroborated by impaling a neurone twice, the first time using potassium acetate in the recording electrode, the second using potassium chloride.

Marked desensitization was found with this response. A dose cycle of 1 dose per 40sec or longer had to be employed to ensure reproducible responses.

FIGURE V.7

GABA RESPONSE

Voltage recordings from a spinal neurone, details as for fig V.1. GABA application was by iontophoresis [15nA, bar]. The response is shown over a range of membrane potentials [Vm]. The reversal potential of this response was circa -58mV. Recordings were made in normal recording medium.

Resting membrane potential -65mV

Scale bars 10mV and 2sec.

Vm

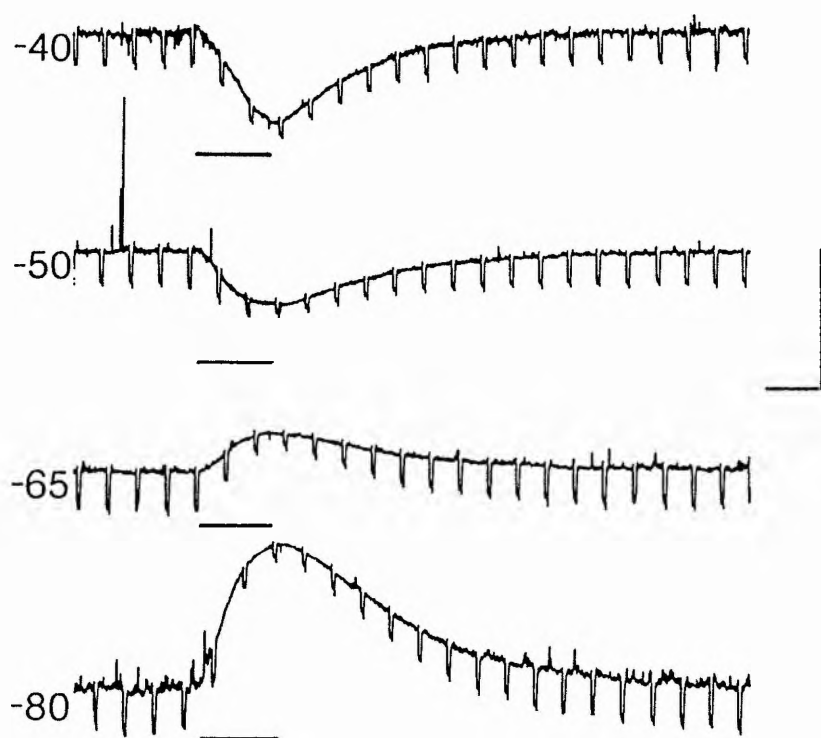


FIGURE V.8

GABA RESPONSE:

AFFECT OF A RAISED INTRACELLULAR CHLORIDE ION CONCENTRATION

Voltage recordings from a spinal neurone, details as for fig V.1. The recording electrode contained 2M potassium chloride. Intracellular chloride was increased by injecting chloride ions out of the recording electrode and into the neurone. Application of GABA was by pressure ejection, [50uM, 100msec arrowheads]. The recordings were made in normal recording medium at a series of membrane potentials [Vm].

Resting membrane potential -55mV

Scale bars 10mV and 2sec.

NOTE: all the spontaneous activity reversed at a similar potential to that of the GABA response.

Vm

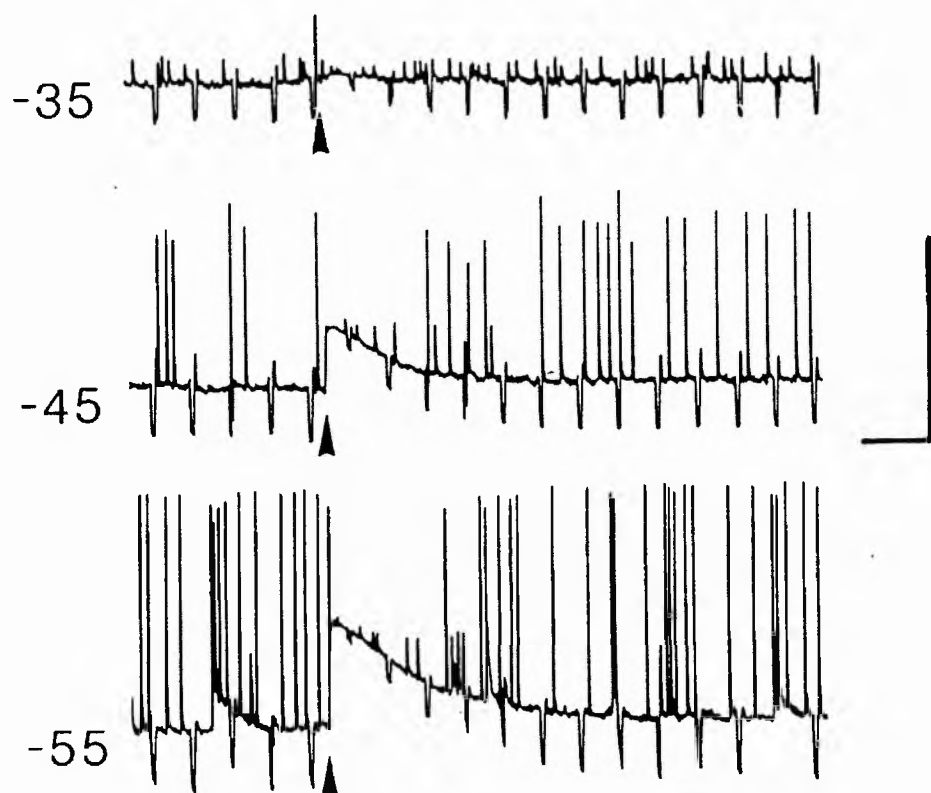


FIGURE V.9

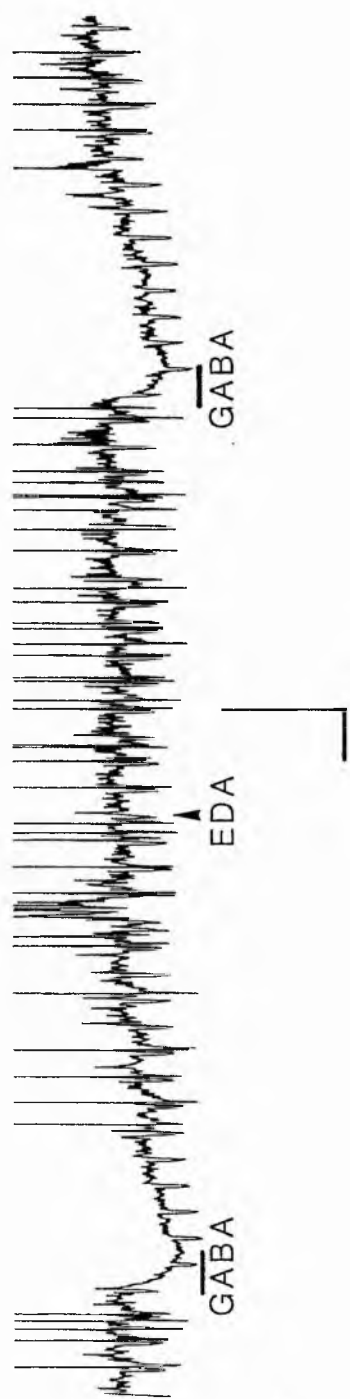
EDA AND GABA

Voltage recording from a spinal neurone, details as for fig V.1. EDA was pressure ejected [100uM, 200msec, indicated by the arrowhead] and GABA was iontophoresed [15nA, bars] onto the neurone. No response was evoked by EDA, whereas the neurone did respond to GABA.

Recording was made in normal recording medium.

Resting membrane potential -52mV

Scale bars 10mV and 2sec.



V.3.3] ETHYLENE-DIAMINE [EDA]

The GABA analogue, EDA was pressure ejected onto 25 and iontophoresed onto 5 neurones, at pipette concentrations of 100uM and 1mM respectively. The cells were from 3 different culture batches. None of these gave a response. GABA was occasionally applied to the neurone in between EDA doses [fig V.9.] to ascertain if desensitization was occurring. It was found that each GABA application evoked a response from the neurone, whereas the EDA did not.

V.4] PEPTIDES

The following peptides were tested on the cultured neurones: met⁵- and leu⁵-enkephalin [Peninsula Laboratories], neurotensin [Peninsula Laboratories], Phe-Met-Arg-Phe-amide [FMRFamide; Peninsula Laboratories] and its analogue Phe-nLeu-Arg-Phe-amide [FnLRFamide; a kind gift from Dr.J.Morley] and glycyl L-glutamine [a kind gift from Dr.D.G.Smyth].

In any culture dish, only one type of response [or mixture of responses] was evoked by a peptide. However, two different peptides [e.g., FMRFamide and an enkephalin] each evoked different responses from neurones in the same culture dish, and occasionally, even from the same neurone [fig V.29].

V.5] ENKEPHALINS

Both excitatory and inhibitory responses were found with enkephalins. However, only rarely was more than one type of response evoked from a neurone by enkephalin. In those neurones, both responses appeared simultaneously, thus making them difficult to resolve. Of 472 neurones which were tested with the enkephalins [pipette concentrations of 10 to 500uM], only 17% [81] gave any direct response. Approximately 75% of the neurones exhibiting enkephalin responses were found together, in small groups. The direct effects were of three types, as described below.

FIGURE V.10

ENKEPHALIN RESPONSES: DECREASE IN INPUT RESISTANCE

Voltage recordings from spinal neurones, details as for fig V.1. Enkephalins applied by pressure ejection, [100uM, 200msec; arrowheads]. Responses shown at a range of membrane potentials [Vm]. The variation in reversal potential between neurones was seen with both enkephalins.

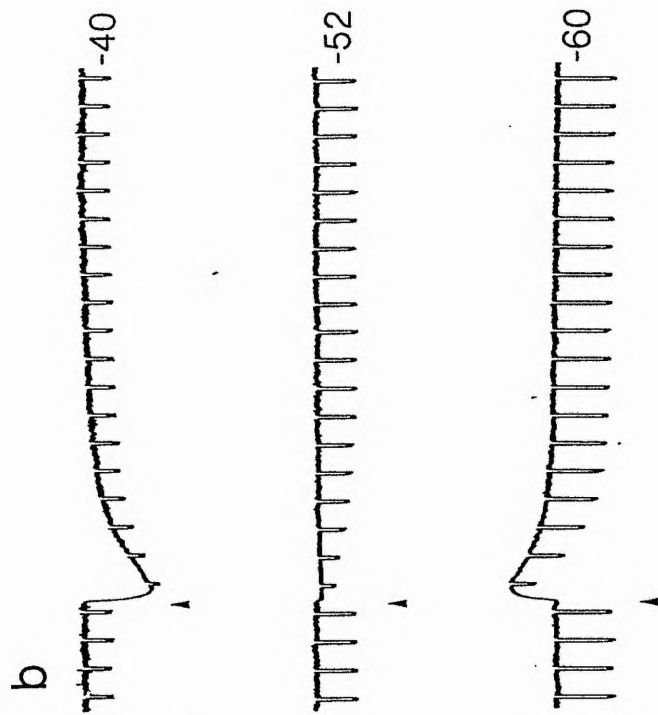
a) Response to leu⁵-enkephalin. Resting membrane potential -58mV.

b) Response to met⁵-enkephalin. Resting membrane potential -52mV.

Recordings were made in high magnesium recording medium.

Scale bars 10mV and 2sec.

Vm



Vm

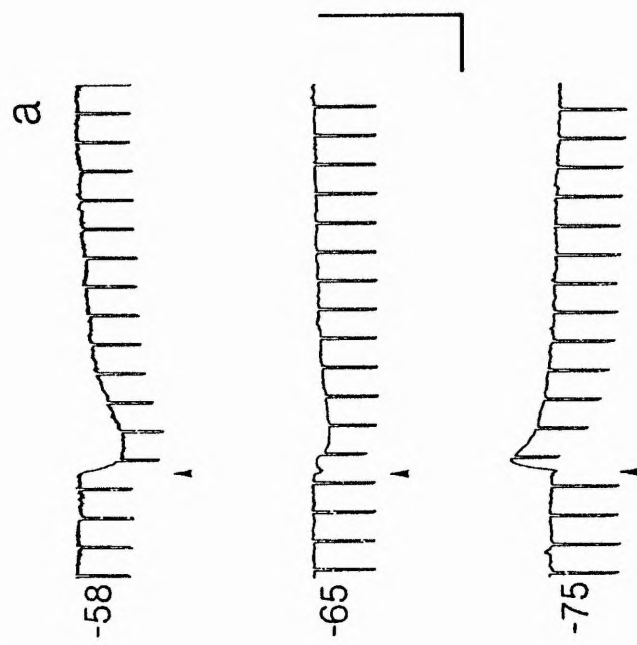


FIGURE V.11

NALOXONE AND A RESPONSE TO ENKEPHALIN: DECREASE IN INPUT RESISTANCE

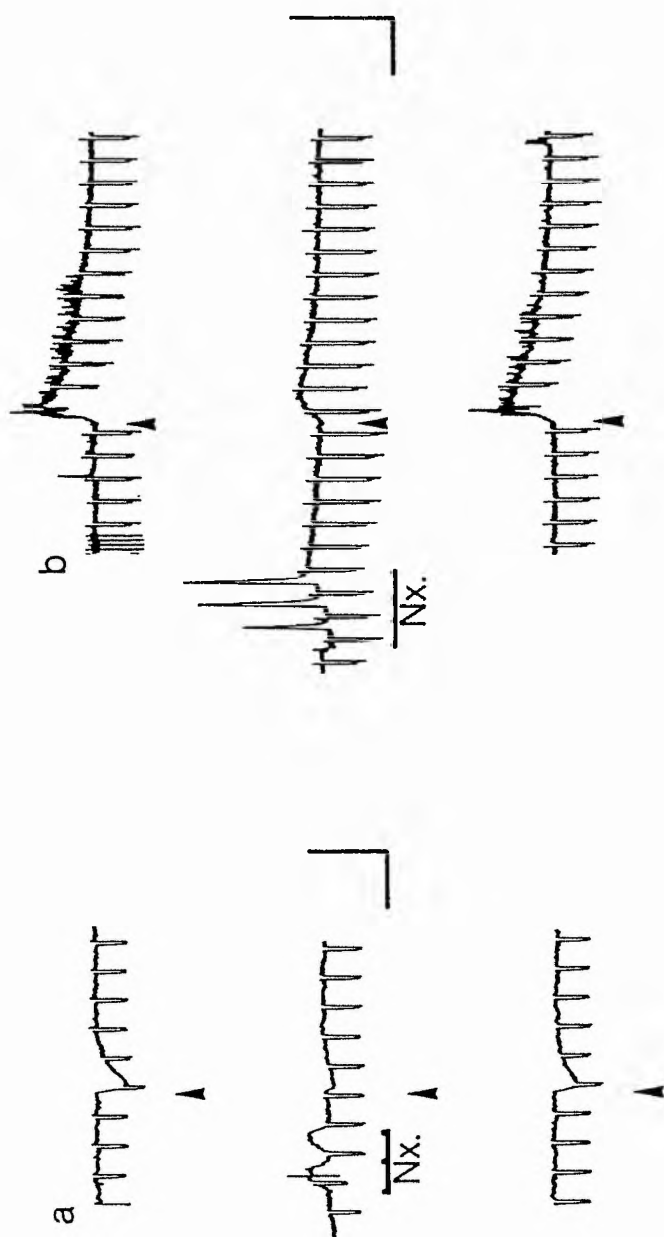
Voltage recordings from spinal neurones, details as for fig V.1. Leu⁵-enkephalin application by pressure ejection, [100uM, 200msec; arrowheads]. Pressure ejection [3 pulses] of naloxone [200uM; bar] from broken micropipettes [tip diameters up to 5um]. Dose cycles for enkephalin administration were: a] 1 per 20sec, b] 1 per 40sec. Scale bars indicate 10mV and 2sec.

Top recording: enkephalin response prior to naloxone. Middle recording: enkephalin response in the presence of naloxone. Bottom recording: post-naloxone control response to enkephalin.

a) Enkephalin response [decrease in input resistance], recordings were made in high magnesium recording medium. Resting membrane potential -52mV.

b) Enkephalin evoked increase in synaptic activity. Recordings were made in normal recording medium. Resting membrane potential -55mV.

NOTE: in both experiments, naloxone also altered the membrane potential and input resistance.



V.5.1] INHIBITORY RESPONSES

These responses were accompanied by a decrease in input resistance. The reversal potentials varied between individual neurones, from -70 to -40mV, fig V.10. Most of the spontaneous activity was suppressed during these responses. This effect was seen in 34 neurones, 42% of those which responded to enkephalin.

After a rapid onset, the response usually faded within 20sec. However, these responses did not show desensitization on repeated application of peptide. No voltage dependence was associated with this response, over the range of membrane potentials from -80 to -40mV. This effect was present in solutions containing high magnesium/low calcium and was attenuated by microperfusing naloxone at concentrations $\leq 100\mu\text{M}$, the latter on 2 occasions [fig V.11].

FIGURE V.12

ENKEPHALIN RESPONSE:

DEPOLARIZATION / DECREASE IN INPUT RESISTANCE

Voltage recordings from a spinal neurone, details as for fig V.1. Leu⁵-enkephalin was applied by pressure ejection [100uM, 200msec, arrowheads]. The response is shown over a range of membrane potentials [Vm]. The extrapolated reversal potential for this response was circa -20mV.

These recordings were made in normal recording medium.

Resting membrane potential -58mV

Scale bars 10mV and 2sec.

Vm

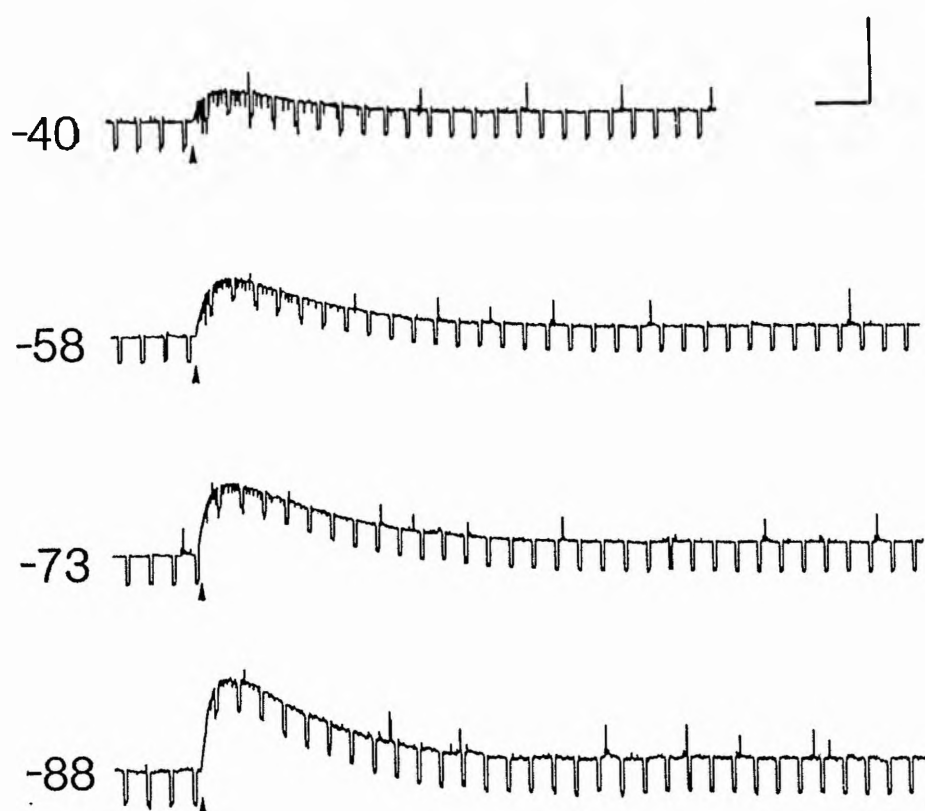


FIGURE V.13

NALOXONE AND AN ENKEPHALIN-EVOKED DEPOLARIZATION / DECREASE IN INPUT RESISTANCE

Voltage recordings from a spinal neurone, details as for fig V.1. Met⁵-enkephalin was applied by pressure ejection [100uM, 150msec, arrowheads]. Naloxone applied by microperfusion from a broken micropipette [100uM, bar].

The recordings were made in normal recording medium.

Resting membrane potential -55mV.

A] control response

B] naloxone microperfusion followed by an enkephalin response

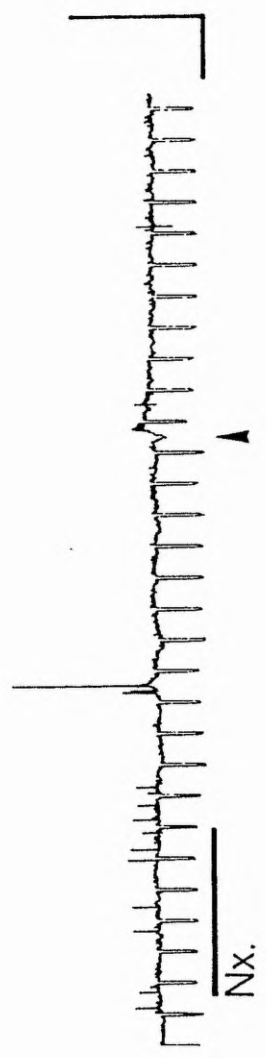
C] wash, control response.

A 40sec dose cycle was used for the enkephalin applications.

Scale bars 10mV and 2sec.



a



b



c

FIGURE V.14

ENKEPHALIN RESPONSE:

DEPOLARIZATION / INCREASE IN INPUT RESISTANCE

Voltage recordings made from a spinal neurone, details as for fig V.1. Met⁵-enkephalin was pressure ejected onto the neurone [100uM, 150msec, arrowheads]. The response is shown over a range of membrane potentials [Vm]. Recordings made in high magnesium recording medium.

Resting membrane potential -65mV

Scale bars 10mV and 2sec.

Vm

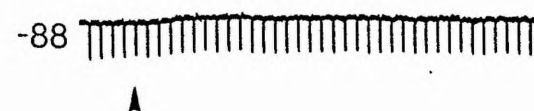


FIGURE V.15

GRAPHICAL REPRESENTATION OF AN ENKEPHALIN-EVOKED
THE DEPOLARIZATION / INCREASE IN INPUT RESISTANCE

These graphs show the relationship between membrane potential [mV] and both the response size [mV] and the resistance change [as a percentage of that prior to enkephalin application]. Resistance change was calculated by comparing the voltage deflections [induced by the constant current pulses] before and during the response. Application of leu⁵-enkephalin was by pressure ejection [400uM, 150msec].

Resting membrane potential of the neurone was -54mV.

Recording made in normal recording medium.

Resistance
change (%)

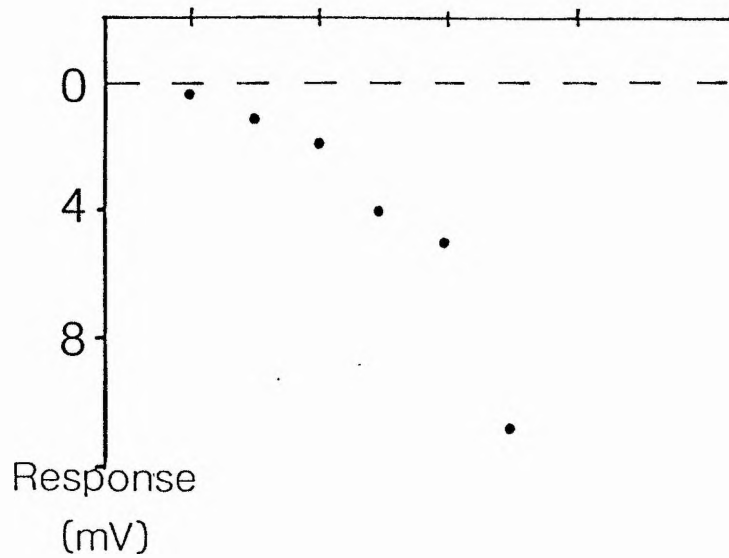
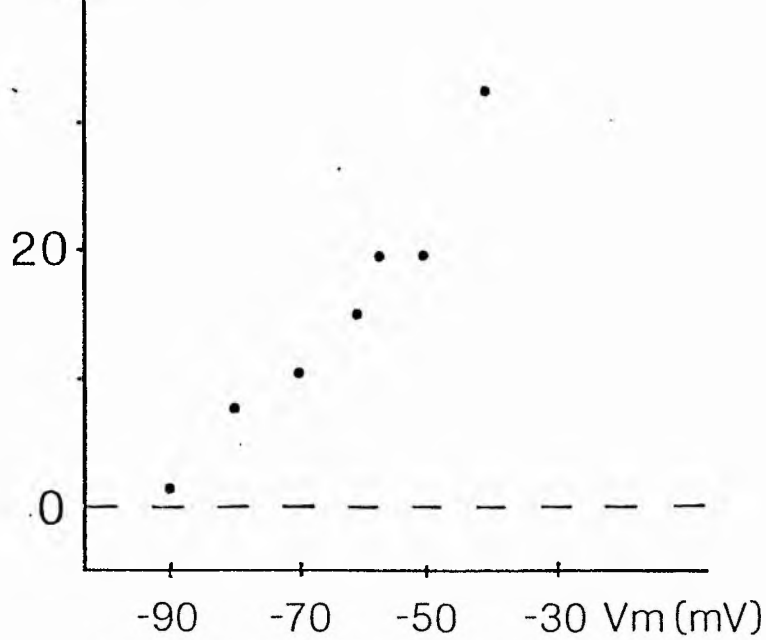


FIGURE V.16

NALOXONE AND THE ENKEPHALIN-EVOKED

DEPOLARIZATION / INCREASE IN INPUT RESISTANCE

Voltage recordings from a spinal neurone, details as in fig V.1. Naloxone was applied by microperfusion from a broken micropipette [200uM, bar]. Leu⁵-enkephalin was pressure ejected onto the neurone [100uM and 200msec, arrowheads]. Dose cycle for enkephalin administration, 1 per 40sec. Recordings made in normal recording medium.

A] control enkephalin response

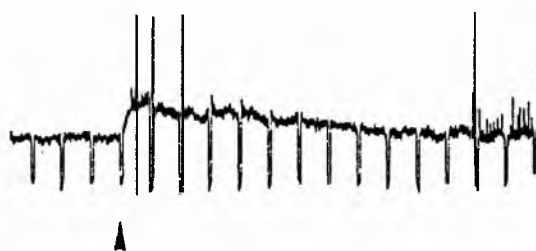
B] naloxone [Nx.] microperfusion prior to enkephalin response

C] post-naloxone control response to enkephalin

The experiments were performed at the resting membrane potential, -60mV.

Scale bars 10mV and 2sec.

a



b



c



V.5.2] EXCITATORY RESPONSES

Two varieties of excitatory response were found which were apparently postsynaptic.

A] Depolarizations accompanied by an apparent decrease in input resistance. The extrapolated reversal potential for these responses was approximately -20mV. This type of response was evoked from 38 neurones; representing 47% of those spinal neurones which responded to enkephalin. The peak was reached usually within 3sec of the pressure pulse, after which the response slowly faded over the following 40sec, fig V.12.

In normal medium, an increase in the incidence of synaptic potentials occurred simultaneously with this type of response, fig V.12. This effect was seen with both met⁵- and leu⁵- enkephalins when either was pressure ejected at concentrations of between 50 and 200uM. In the presence of $\leq 100\text{uM}$ naloxone, microperfused from a broken micro-pipette, the response was reversibly attenuated in 3 of 4 experiments, fig V.13; on the other occasion the neurone died before the response was able to recover. This response showed no voltage dependence over the potential range -90 to -40mV.

B] Depolarizations accompanied by an apparent increase in input resistance. At resting potential these responses required in excess of 5sec to reach a maximum and then faded gradually over the next minute, fig V.14. These responses appeared to show a marked voltage dependence; at potentials more negative than -80mV, very little if any membrane effects were found. However, at depolarized potentials

the response became larger in size and longer in duration. The total number of spinal neurones giving this response was 9, only 11% of the spinal neurones which responded to the enkephalins.

Figure V.15 is a graphical representation of the response parameters at different membrane potentials. These changes were measured over the potential range -90 to -40mV. The extrapolated reversal potential for this response was between -90 and -75mV [the voltage dependence of this response interfered with extrapolations made to determine the reversal potential of these responses]. Increases in the number of both synaptic potentials and action potentials were associated with this type of response when the recordings were being made in normal recording medium. These responses were also present in high magnesium/low calcium containing solutions, which infers they were postsynaptic in origin. When challenged with microperfused naloxone at concentrations $\leq 100\mu\text{M}$, the response was reversibly attenuated in 2 neurones [cf. fig V.16].

V.5.3] EFFECTS OF NALOXONE

In 50% of the experiments where naloxone was microperfused onto a neurone it produced a transient alteration of the membrane potential and input resistance, fig V.11. These effects were depolarizations and increases in input resistance. The duration and size of the response varied between neurones.

FIGURE V.17

ENKEPHALIN RESPONSE: DECREASE IN INPUT RESISTANCE

MET⁵-ENKEPHALIN

Voltage recordings from a spinal neurone, details as for fig V.1. Enkephalin was applied by pressure ejection [100uM, 200msec, arrowheads]. The response is shown over a range of membrane potentials [Vm]. Recordings were made in normal recording medium. Resting membrane potential -57mV.

Scale bars 10mV and 2sec [the latter part of the middle recording was made at a slower speed, horizontal scale bar 10s].

Vm

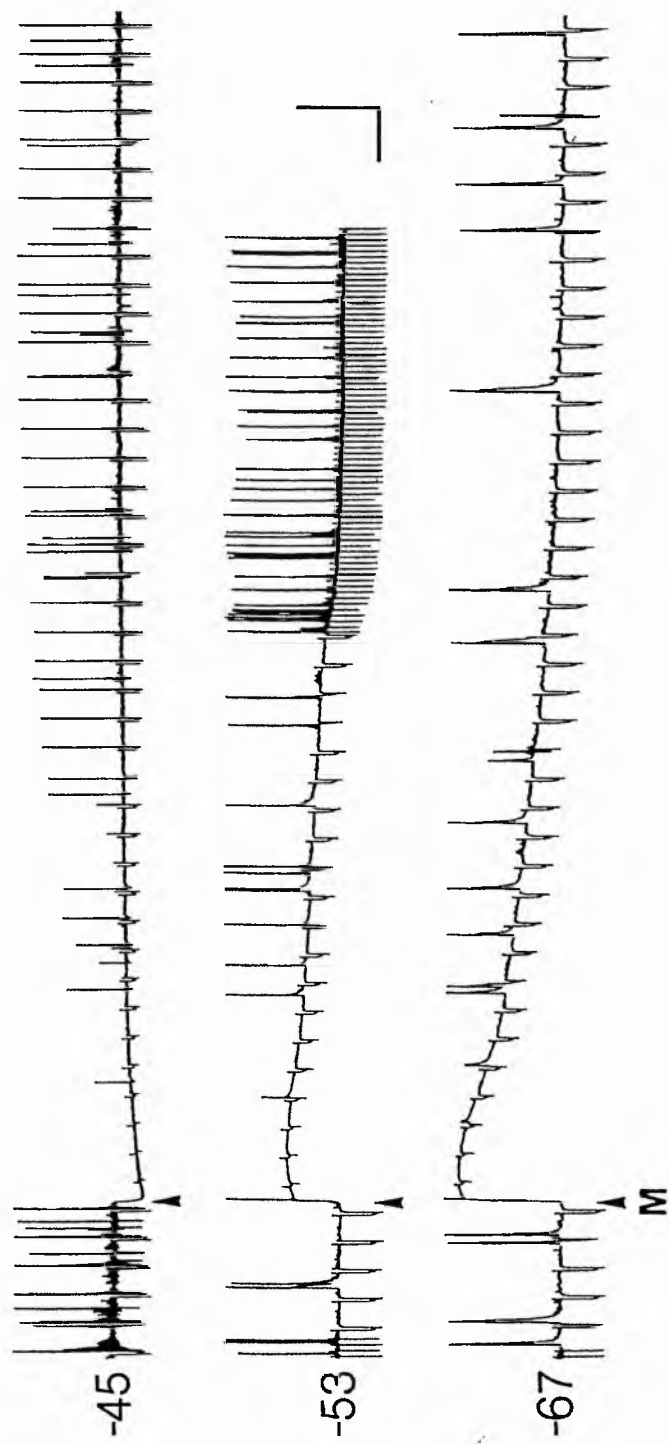


FIGURE V.18

ENKEPHALIN RESPONSE: DECREASE IN INPUT RESISTANCE

LEU⁵-ENKEPHALIN

Voltage recordings from a spinal neurone, details as for fig V.1. Enkephalin was applied by pressure ejection [100uM, arrowheads]. The period of pressure ejection varied [A, 200msec; B, 400msec; C, 800msec; D, 1600msec]. Recordings were made in normal recording medium.

Resting membrane potential -54mV.

Scale bars 10mV and 2sec.

NOTE: the prolonged time course of this response and compare this with the response, from the same spinal neurone, to met⁵-enkephalin [fig V.19, also compare the doses used].



a



b



c



L



d

L

FIGURE V.19

ENKEPHALIN RESPONSES: MET⁵- ENKEPHALIN

Voltage recordings from a spinal neurone, details as for fig V.1. Enkephalins were applied separately by pressure ejection [100uM, arrowheads]. The period of ejection used for each differed. Recordings were made in normal recording medium and at the resting membrane potential [-54mV].
Scale bars 10mV and 2sec.

A] Met⁵-enkephalin, dose cycle of 1 per 40sec. Doses used were: top, 50msec; middle, 100msec; bottom, 200msec.

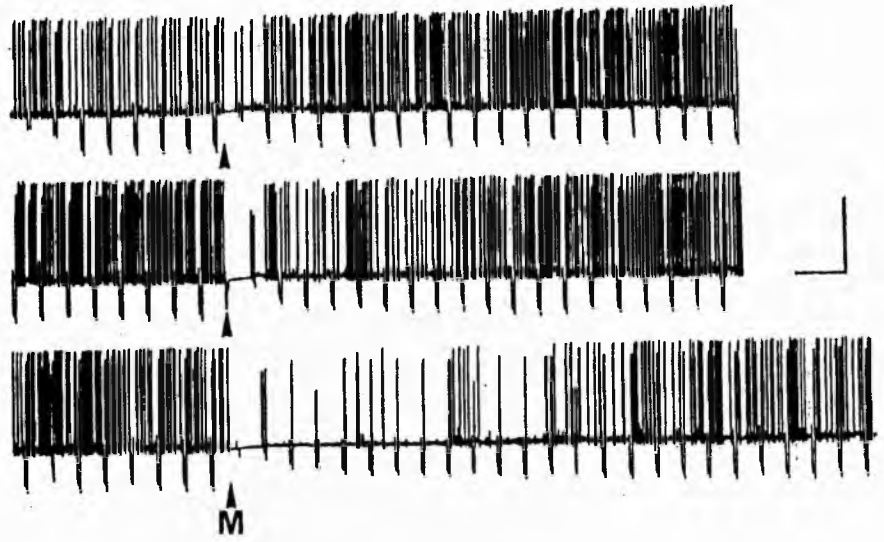
INTERACTION BETWEEN MET⁵- AND LEU⁵- ENKEPHALINS

B] Affect of prior application of leu⁵-enkephalin [L, 400msec] on the response to a 200msec dose of met⁵-enkephalin [M]. The neurone had hyperpolarized slightly by this time, hence the lower rate of activity. Dose cycle for the met⁵-enkephalin was 1 per 40sec.

C] A diagrammatic representation showing the relative positions of the drug application electrodes [M,L] to the cell and the recording electrode [R].

NOTE: both drug pipettes were rarely in position at the same time and the pressure used to apply the peptides remained constant.

a



b



c

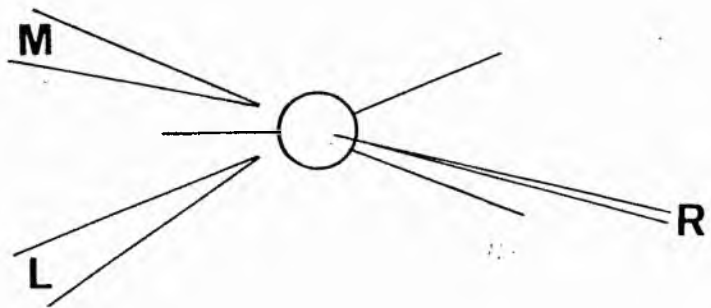


FIGURE V.20

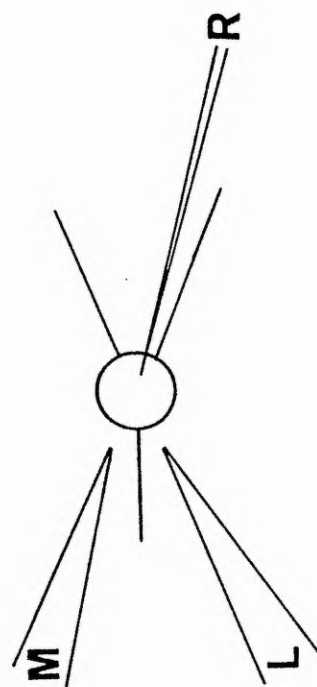
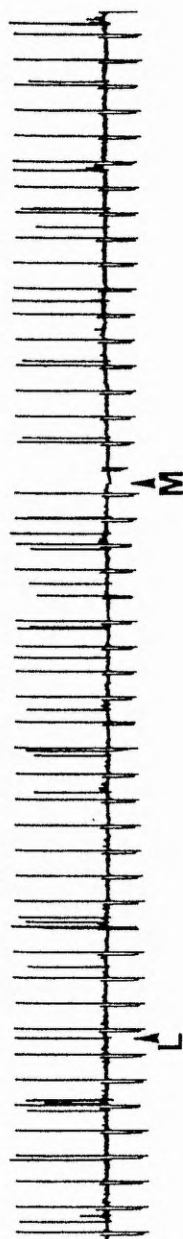
MET⁵- AND LEU⁵- ENKEPHALIN RESPONSES FROM THE SAME NEURONE

Voltage recording from a spinal neurone, details as for fig V.1. Met⁵- and leu⁵- enkephalins [M and L, respectively] were each applied by pressure ejection [100uM, arrowheads]. The electrodes used were of similar tip diameters, being made with the same "pull". Dose cycle for the met⁵-enkephalin was 1 per 40sec. Met⁵-enkephalin [at 200msec duration] was applied during a response which had been evoked by leu⁵-enkephalin. These recordings were from the same neurone as those shown in figs V.18 and V.19.

Resting membrane potential, at the time of this recording, -55mV.

Scale bars 10mV and 2sec.

The diagrammatic representation shows the relative positions of the electrodes used in this experiment. [M] and [L] indicate the drug pipettes, [R] the recording electrode.



V.5.4] SEPARATE APPLICATION OF MET⁵- AND LEU⁵- ENKEPHALINS
TO THE SAME NEURONE

Both met⁵- and leu⁵- enkephalins were tested on 32 enkephalin-responsive spinal neurones in culture. All of those neurones showed responses associated with a decreased input resistance. On 20 occasions, only one of the opioid peptides [either met⁵- or leu⁵- enkephalin; 17 and 3 neurones respectively], evoked a response from the neurone. Both of these enkephalins evoked a response from the remaining 12 neurones. When tested on 8 of these 12 neurones, the enkephalins evoked responses with similar characteristics.

On the remaining 4 neurones the enkephalins produced responses of a similar nature but the duration and "potency" differed greatly [cf. fig V.17 and fig V.18]. The responses from all 4 neurones were inhibitory, being hyperpolarizations accompanied by a decrease in input resistance. In these 4 neurones, the administration of the more potent met⁵-enkephalin during the response of the less potent leu⁵-enkephalin resulted in a reduced overall response to the met⁵-enkephalin, figs V.19 and 20. This effect was reproducible and seen in two neurones.

FIGURE V.21

ENKEPHALIN: AMINO ACID MODULATION ?

Voltage recordings from spinal neurones, details as for fig V.1. Each series was constructed from responses to consecutive amino acid applications.

Top recording: control response to amino acid.

Middle recording: amino acid response following leu⁵-enkephalin microperfusion [200uM, bar].

Lower recording: amino acid response, post-enkephalin.

AMINO ACIDS

- a) GABA application by iontophoresis [10nA, thin line]. Dose cycle, 1 per 40sec.

Resting membrane potential -56mV.

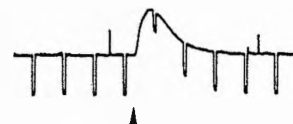
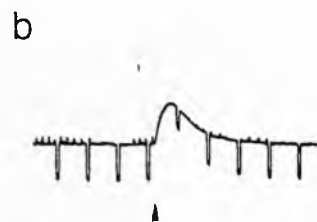
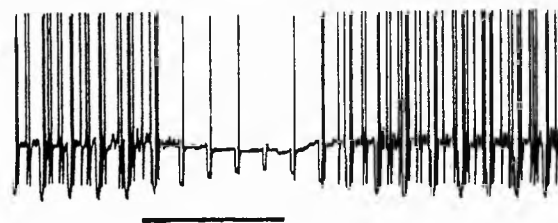
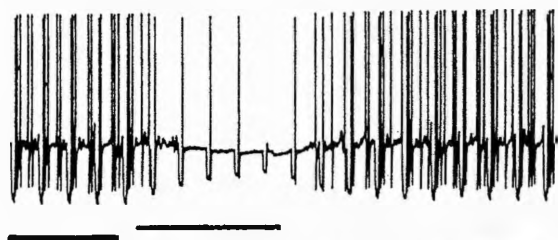
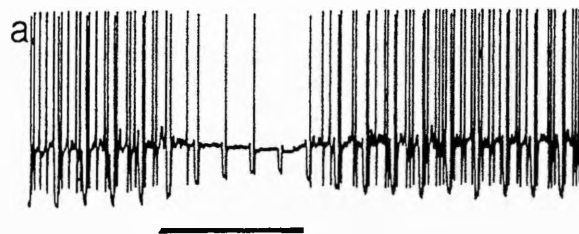
- b) Glycine application by pressure ejection [50uM, 250msec]. Dose cycle, 1 per 40sec.

Resting membrane potential -62mV.

- c) L-Glutamate application by pressure ejection [50uM, 100msec]. Dose cycle, 1 per 60sec.

Resting membrane potential -58mV.

Scale bars for a) and b), 10mV and 2sec and for c) 10mV and 10sec.



C



V.5.5] MODULATION OF AMINO ACID RESPONSES

V.5.5.1] GABA and GLUTAMATE

Using the method already described [section III.7], GABA and L-glutamate responses were obtained in the presence of either met⁵- or leu⁵- enkephalin. These were compared with GABA or L-glutamate responses obtained before and after the application of opioid peptide solution. No difference was found between these amino acid responses prior to, during or post enkephalin application, fig V.21. Enkephalin concentrations in the perfusion pipette were between 1 and 400uM.

V.5.5.2] GLYCINE

The same experimental protocol was used as described in section III.7. When leu⁵-enkephalin concentrations of >400uM were used the glycine response was occasionally depressed [2 out of 5 tests], fig V.21. However, no change in the glycine response was seen at concentrations of leu⁵-enkephalin lower than 400uM.

FIGURE V.22

FMRFamide RESPONSE: DECREASED INPUT RESISTANCE

Voltage recordings from spinal neurones, details as for fig V.1.

FMRFamide was applied by pressure ejection:

- a) 200uM, 200msec at the arrows,
- b) 100uM, 150msec at the arrows.

Shown over a range of membrane potentials [Vm].

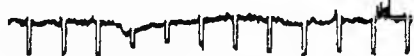
- a) Recording made in normal recording medium. Resting membrane potential -52mV.
- b) Recording made in high magnesium recording medium. Resting membrane potential -55mV.

Scale bars 10mV and 2sec.

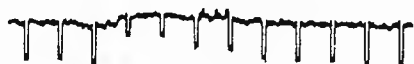
a

Vm

-40



-45



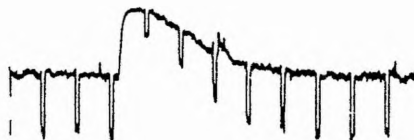
-50



-55



-60



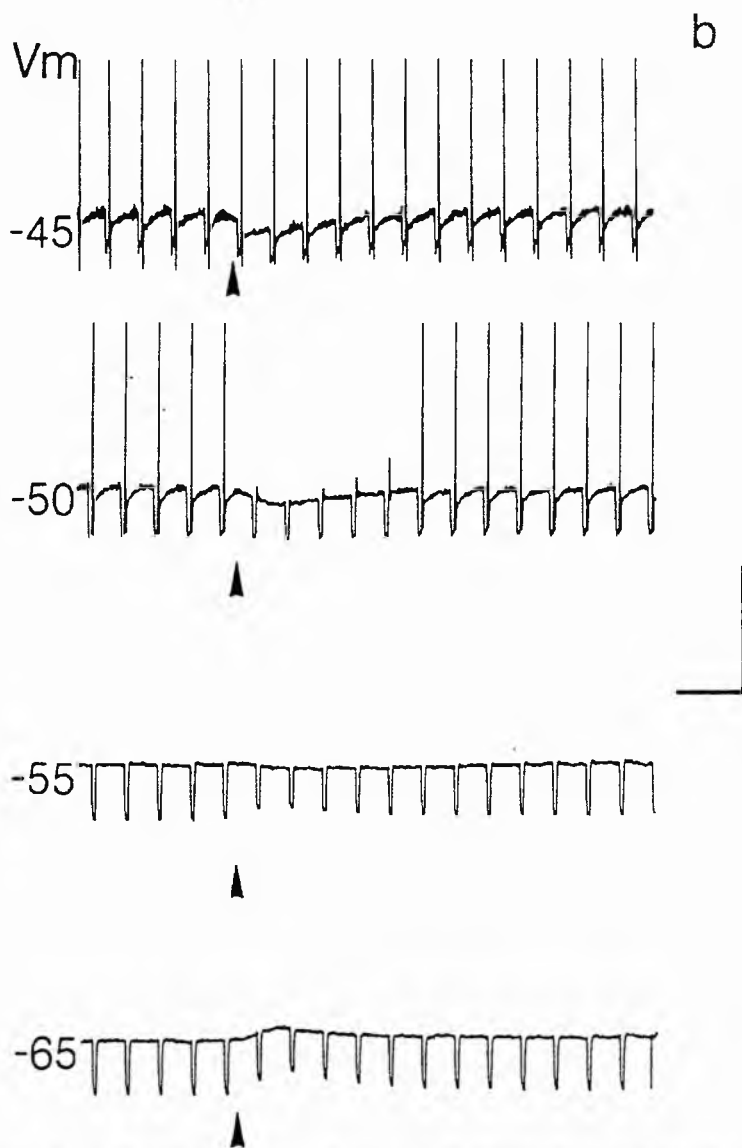


FIGURE V.23

FMRFamide RESPONSE: DECREASED INPUT RESISTANCE

Histogram showing the variation in reversal potential seen between different neurones.

Vertical axis: number of neurones.

Horizontal axis: reversal potential [mV].

NOTE: those neurones bathed in high magnesium recording medium, which exhibited this type of response, had a similar variation in reversal potential to those bathed in normal recording medium.

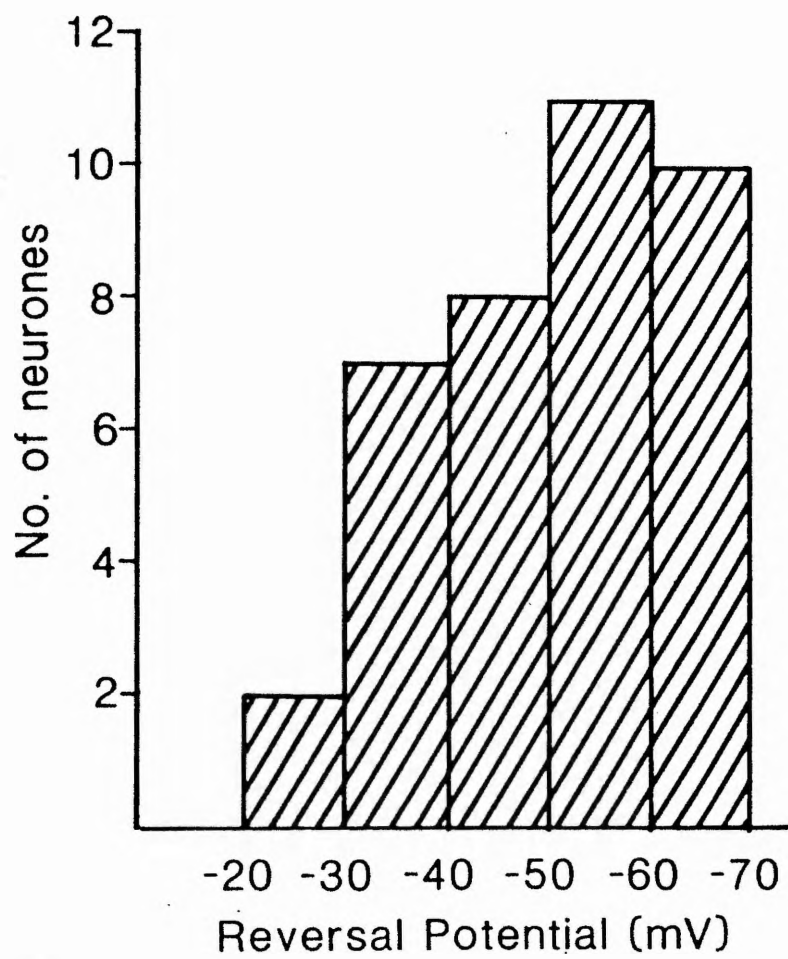


FIGURE V.24

FMRFamide RESPONSE: DECREASED INPUT RESISTANCE
AFFECT OF ALTERING EXTRACELLULAR SODIUM OR CHLORIDE CONCENTRATION.

Voltage recordings from spinal neurones, details as for fig V.1.

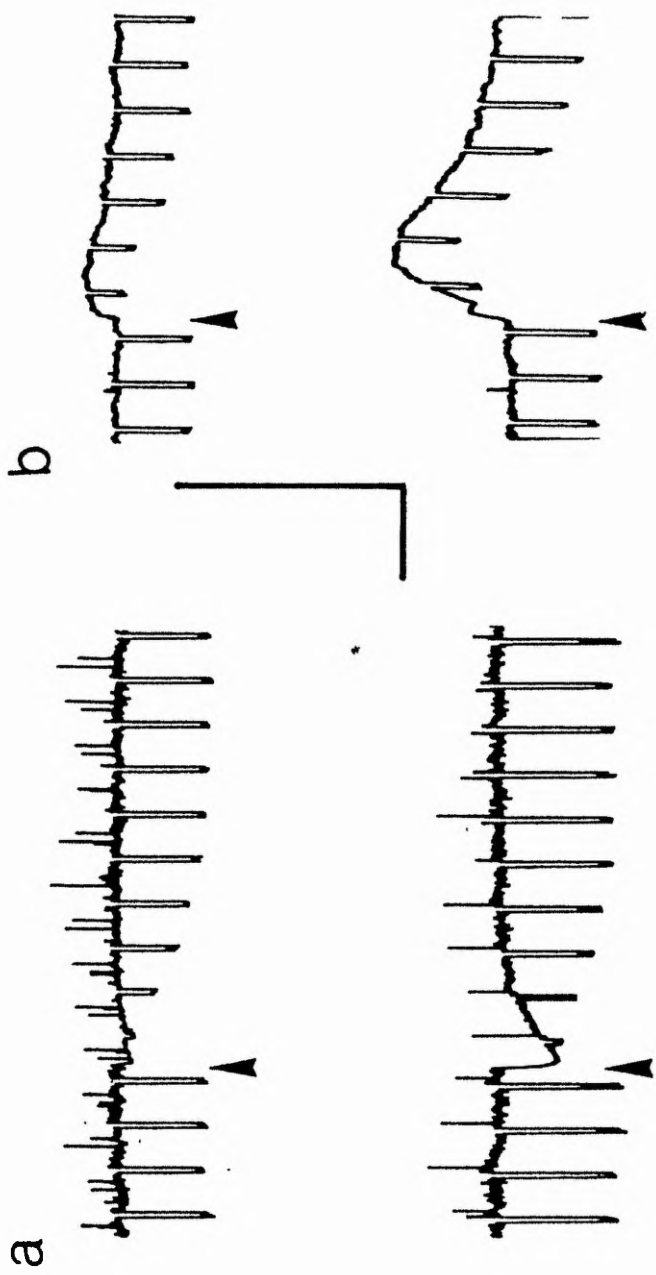
a) Top recording shows the control response in normal recording medium, sodium 132mM. Bottom recording shows the response 10 min. after the start of perfusion with low sodium [66mM] in normal recording medium [choline exchanged for sodium].

Resting membrane potential -54mV

b) Top recording shows the control FMRFamide response in normal recording medium, chloride 156mM. Bottom recording shows the response 7 min. after the start of perfusion with low chloride [75mM] in normal recording medium [isethionate exchanged for chloride].

Resting membrane potential -58mV

Scale bars, 10mV and 2sec.



V.6] FMRFamide AND FnLRFamide

Two types of response were found with FMRFamide, however, only one of these was seen with FnLRFamide. From 420 neurones tested with these peptides [at pipette concentrations of 10 to 500uM], 80 [19%] gave a response. In 30% of the responsive neurones, mixtures of the actions described below appeared either, simultaneously or overlapped, fig V.27. On these occasions, no specific area on the neurone was found which preferentially gave either of the responses.

V.6.1] INHIBITORY RESPONSES

These responses were found in 41 neurones with FMRFamide [51% of those which gave a response to FMRFamide], and none with FnLRFamide. They were associated with a decrease in input resistance, fig V.22. Reversal potentials varied between neurones, over the range -70 to -20mV, fig V.23, and had a rapid onset and offset. No voltage dependence was seen over the membrane potential range -80 to -40mV. No desensitization was associated with these responses after repeated or prolonged application of peptide.

The reversal potentials combined with the variation in reversal potentials between neurones which produced this type of response suggested the involvement of a mixture of ionic species. When tested, these responses showed a sensitivity to changes in either the extracellular chloride ion or the extracellular sodium ion concentrations, fig V.24. Alterations in extracellular potassium ion concentration did not affect this type of response.

FIGURE V.25

FMRFamide RESPONSE:

DEPOLARIZATION / INCREASED INPUT RESISTANCE

Voltage recordings from a spinal neurone, details as for fig V.1. FMRFamide was applied by pressure ejection [100uM, 300msec, arrowheads]. Response shown over a range of membrane potentials [Vm] and recorded in normal recording medium.

Resting membrane potential -65mV

Scale bars 10mV and 2sec.

Vm

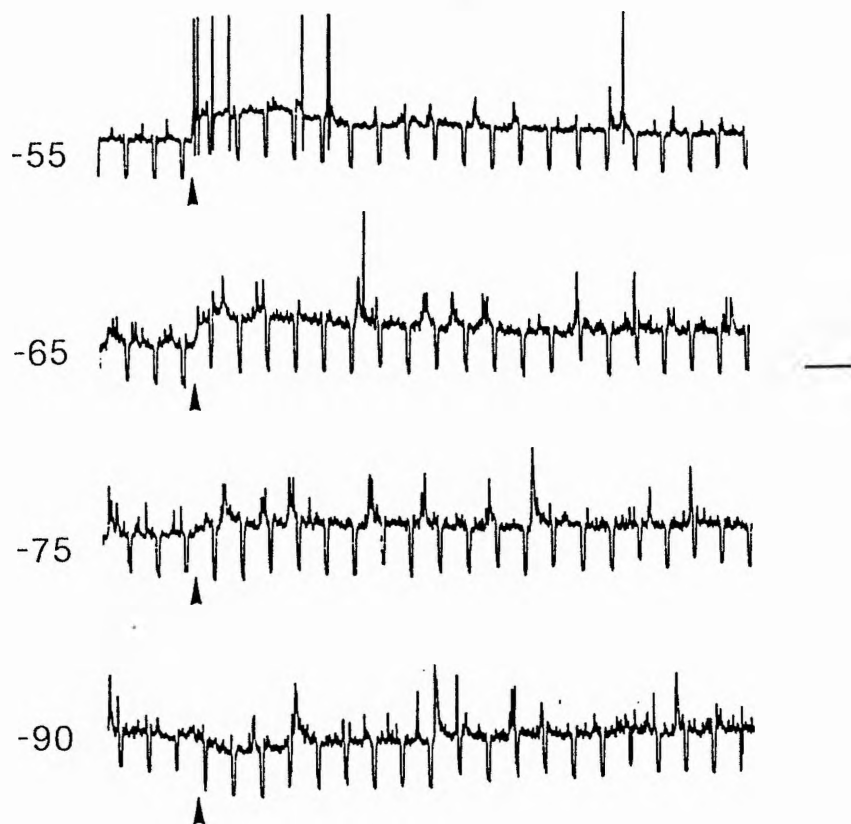


FIGURE V.26

FMRamide-EVOKED DEPOLARIZATION/ INCREASED INPUT RESISTANCE:

AFFECT OF ALTERING EXTRACELLULAR POTASSIUM CONCENTRATION

Voltage recordings from a spinal neurone, details as for fig V.1. FMRamide was applied by pressure ejection [100uM, 500msec; arrowheads].

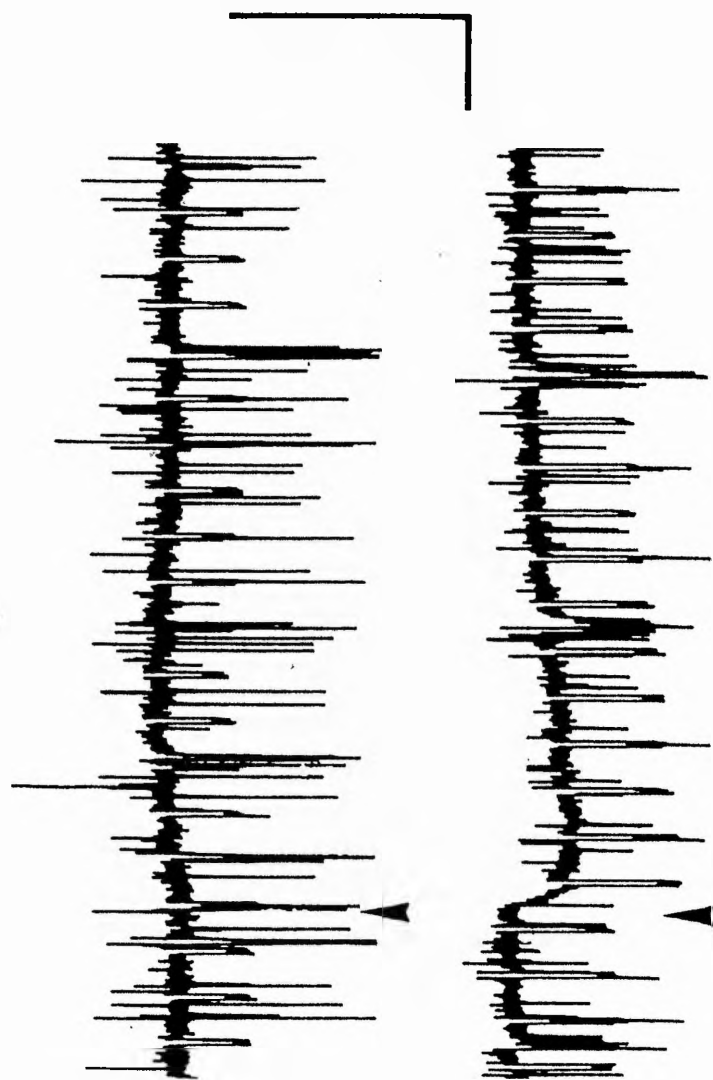
Upper recording: the control response in normal recording medium, potassium 5.8mM.

Lower recording: the response 5 min. after starting perfusion with a high potassium concentration [20mM] in normal recording medium [substitution of potassium for sodium]. A re-adjustment to this level was made by current injection through the recording electrode.

Estimated change in reversal potential was +33mV.

Resting membrane potential -55mV.

Scale bars 10mV and 2sec.



V.6.2] EXCITATORY RESPONSES

These responses were depolarizations which were accompanied by an increased input resistance [fig V.25]. Both FMRFamide and FnlRFamide evoked these responses from the cultured neurones. No voltage sensitivity was found over the range -90 to -45mV . These responses were of a slow onset [between 3 and 10sec to peak] and persisted for up to 1.5 min. This type of response was evoked from 39 neurones with FMRFamide [49% of those which responded to FMRFamide] and 3 of the 8 neurones tested with FnlRFamide. The reversal potentials of these responses were approximately -85mV . No desensitization was seen after repeated or prolonged application of peptide.

A reversal potential of -85mV implied the involvement of potassium ions. To test this possibility, the extracellular potassium ion concentration was increased [from 5.8mM to 20mM , potassium was exchanged for sodium]. As expected, the reversal potential of the response became more positive, fig V.26. An experiment in which the sodium ion concentration was altered had negligible effects on this response and the reversal potential.

FIGURE V.27

FMRamide RESPONSE: BIPHASIC

Voltage recordings from a spinal neurone, details as in fig V.1. FMRamide was applied by pressure ejection [100uM, 100msec, arrowheads]. Response shown at two different membrane potentials [-45mV and -60mV]. Recordings made in high magnesium recording medium.

Resting membrane potential -60mV.

Scale bars 10mV and 2sec.

Vm

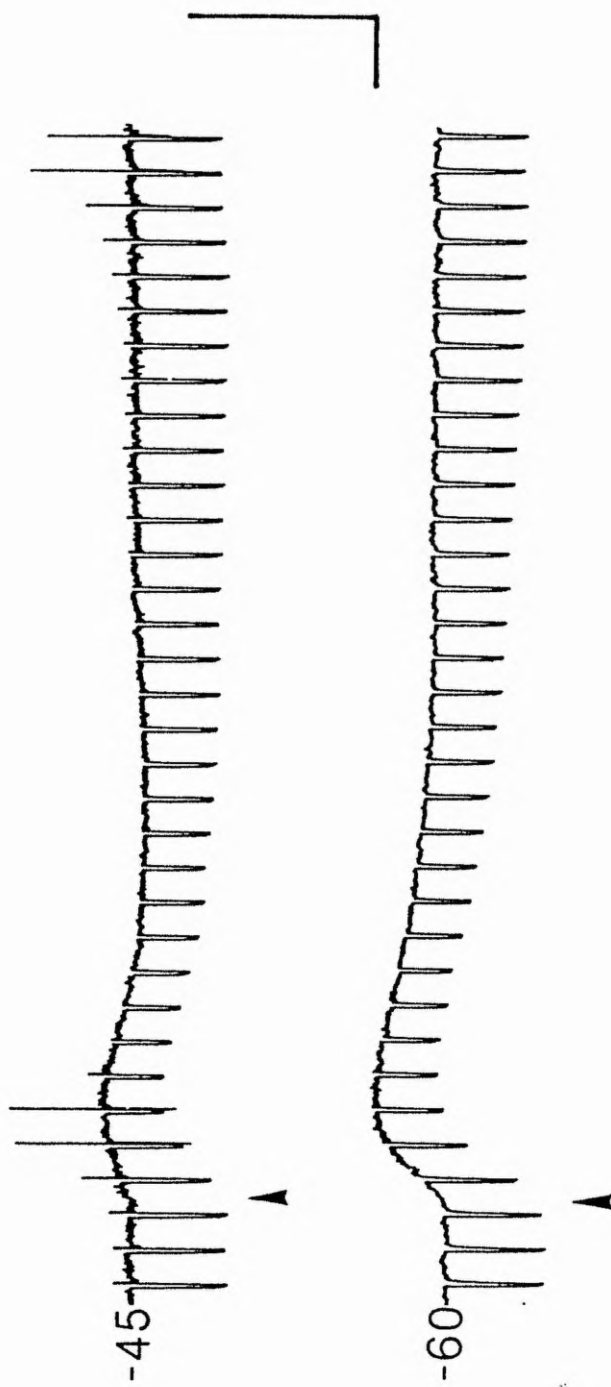


FIGURE V.28

FMRFamide: AMINO ACID MODULATION ?

Voltage recordings made from spinal neurones, details as in fig V.1. FMRFamide applied by microperfusion, [100uM, thick bar]. Scale bars: a] and b] 10mV and 2sec, for c] 15mV and 5sec.

Top recording: control.

Middle recording: FMRFamide followed by amino acid.

Bottom recording: control amino acid response, post-peptide application.

AMINO ACIDS

a) GABA applied by iontophoresis [15nA, thin bar]. Resting membrane potential -52mV.

b) Glycine applied by pressure ejection [100uM, 150msec, arrowheads].

Resting membrane potential -50mV.

c) L-Glutamate applied by pressure ejection [100uM, 150msec, arrowheads].

Resting membrane potential -57mV.

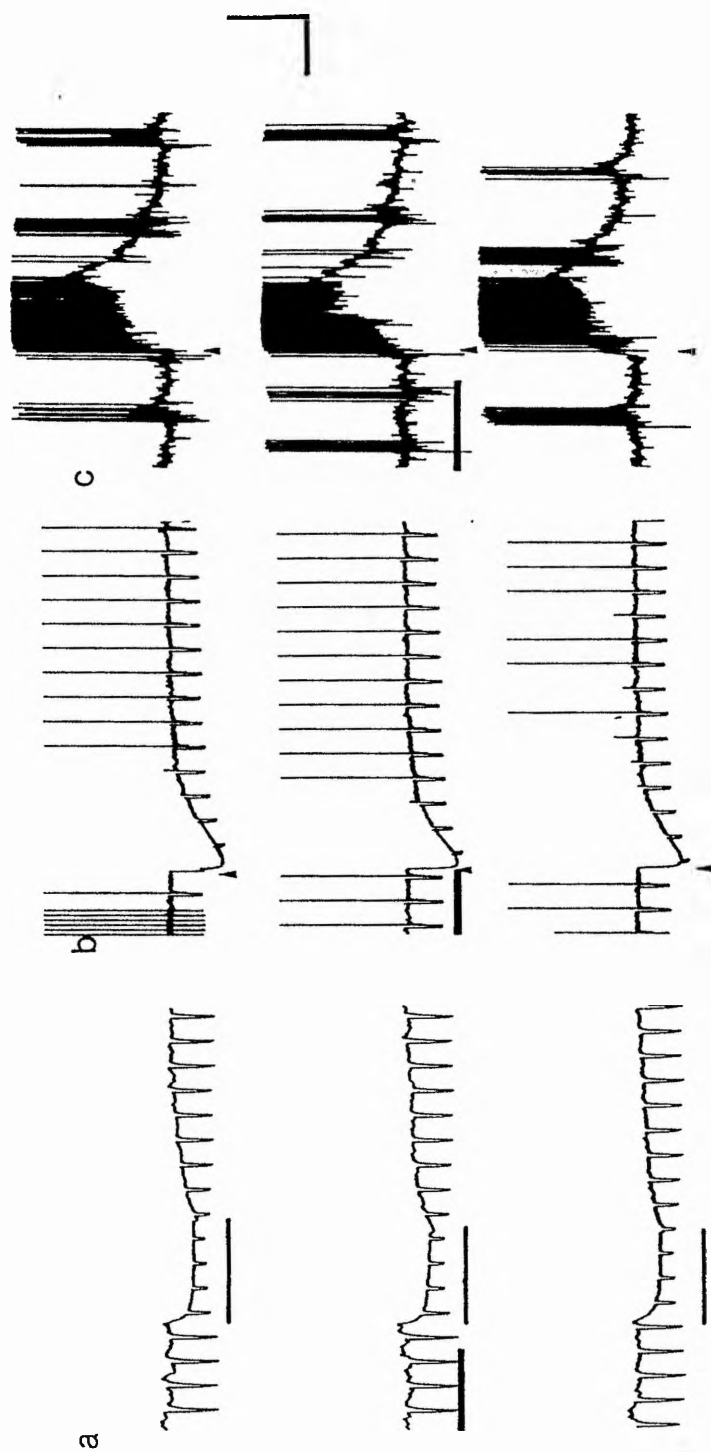


FIGURE V.29

RESPONSES TO FMRFamide AND ENKEPHALIN FROM THE SAME NEURONE

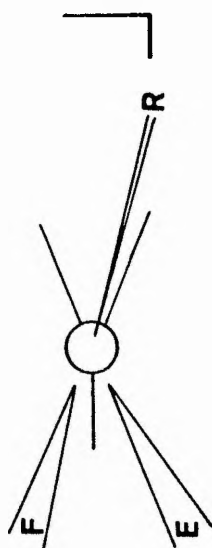
Voltage recordings from a spinal neurone, details as for fig V.1. Both FMRFamide and met⁵-enkephalin were applied by pressure ejection [200uM, 200msec ejection time, arrowheads].

Upper recording shows the response evoked by FMRFamide.

Lower recording shows the response evoked by met⁵-enkephalin.

Resting membrane potential -58mV. Scale bars 10mV and 2sec.

The diagrammatic representation shows the relative positions of the recording [R] electrode, the drug pipettes [F and E] around the neurone.



V.6.3] MODULATION OF AMINO ACID RESPONSES

Experiments were made employing the same protocol as used with the enkephalins, section III.7. No changes to the size or duration of the GABA, glycine or L-glutamate responses were found in the presence of FMRFamide, fig V.28.

V.6.4] RESPONSES TO FMRFamide AND ENKEPHALIN FROM THE SAME NEURONE

Of 22 neurones tested with both FMRFamide and met⁵-enkephalin only 2 gave a response to both peptides. In each case, the response to FMRFamide was a depolarization associated with an increased input resistance. The response evoked by met⁵-enkephalin was also a depolarization, however, these were accompanied by a decreased input resistance. The difference in time course between these 2 peptide responses is illustrated in figure V.29.

V.7] NEUROTENSIN

From over 200 neurones tested with neurotensin [pipette concentrations of between 10 and 100uM], only 5 gave any direct, postsynaptic response. Those five neurones were all found in the same culture dish, situated within 100um of each other. Only one type of response was evoked by neurotensin which appeared to be postsynaptic. These responses were of an inhibitory nature.

FIGURE V.30

NEUROTENSIN RESPONSE: HYPERPOLARIZATION/
DECREASED INPUT RESISTANCE

Voltage recordings from a spinal neurone, details as for fig V.1. Neurotensin was applied by pressure ejection [100uM, 150msec, arrowheads]. Response shown over a range of membrane potentials [Vm]. Recordings were made in normal recording medium.

Resting membrane potential -55mV.

Scale bars 10mV and 2sec.

Vm

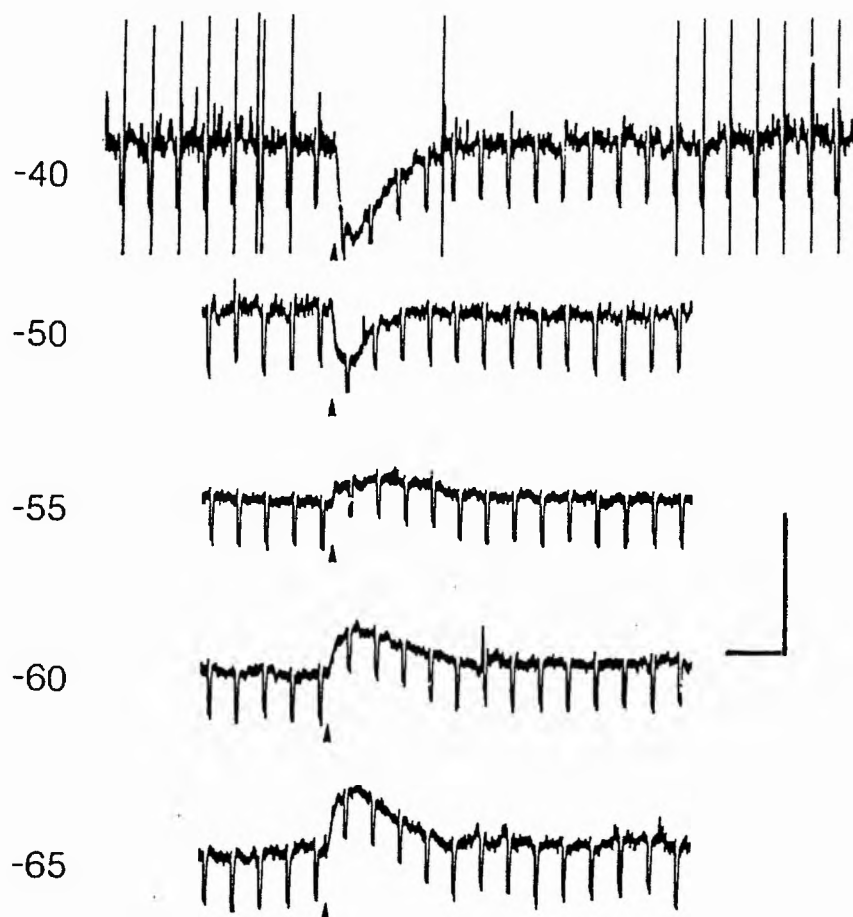


FIGURE V.31

NEUROTENSIN RESPONSE:

AFFECT OF ALTERING THE EXTERNAL POTASSIUM CONCENTRATION

Voltage recordings made from a spinal neurone, details as for fig V.1. Neurotensin was applied by pressure ejection [100uM, 200msec, arrowheads]. Responses are shown at a range of membrane potentials [Vm].

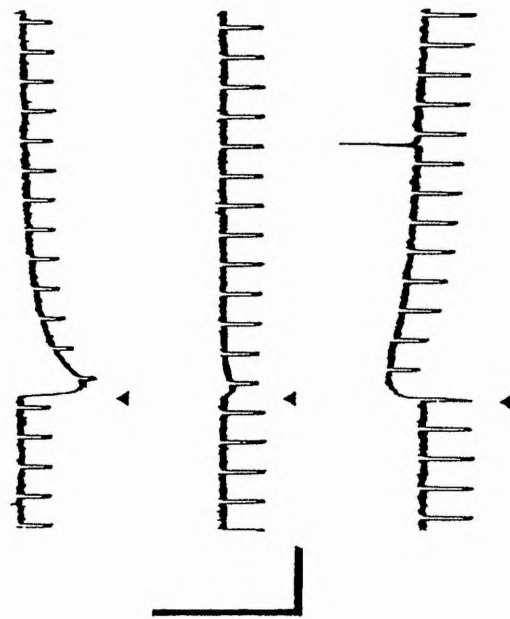
- a) Responses when neurone was bathed in high magnesium/ normal potassium [5.8mM] recording medium.
- b) Response when neurone was bathed in high magnesium/ low potassium [2mM] recording medium.

Resting membrane potential -60mV.

Scale bars 10mV and 2sec.

Vm

a



b

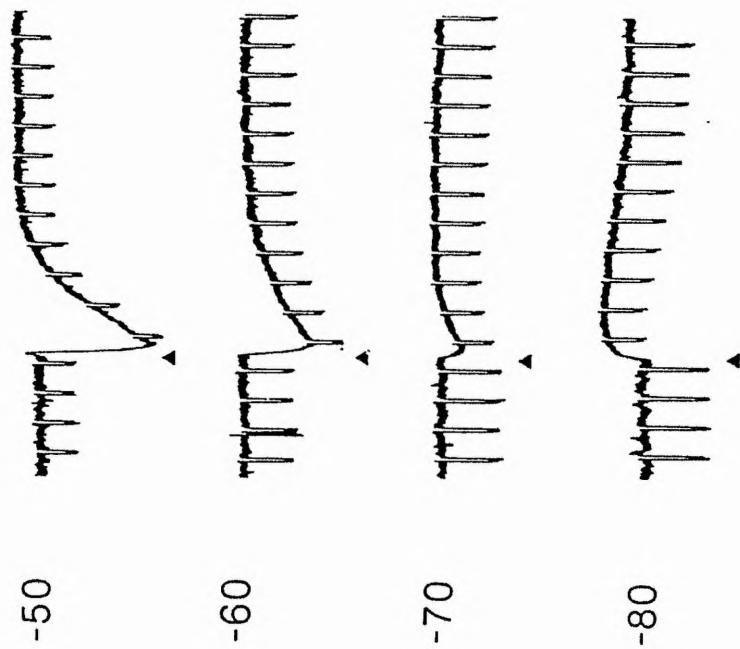


FIGURE V.32

NEUROTENSIN RESPONSE: HYPERPOLARIZATION/
DECREASED INPUT RESISTANCE

A graphical representation of the affect of altering
external potassium ion concentration.

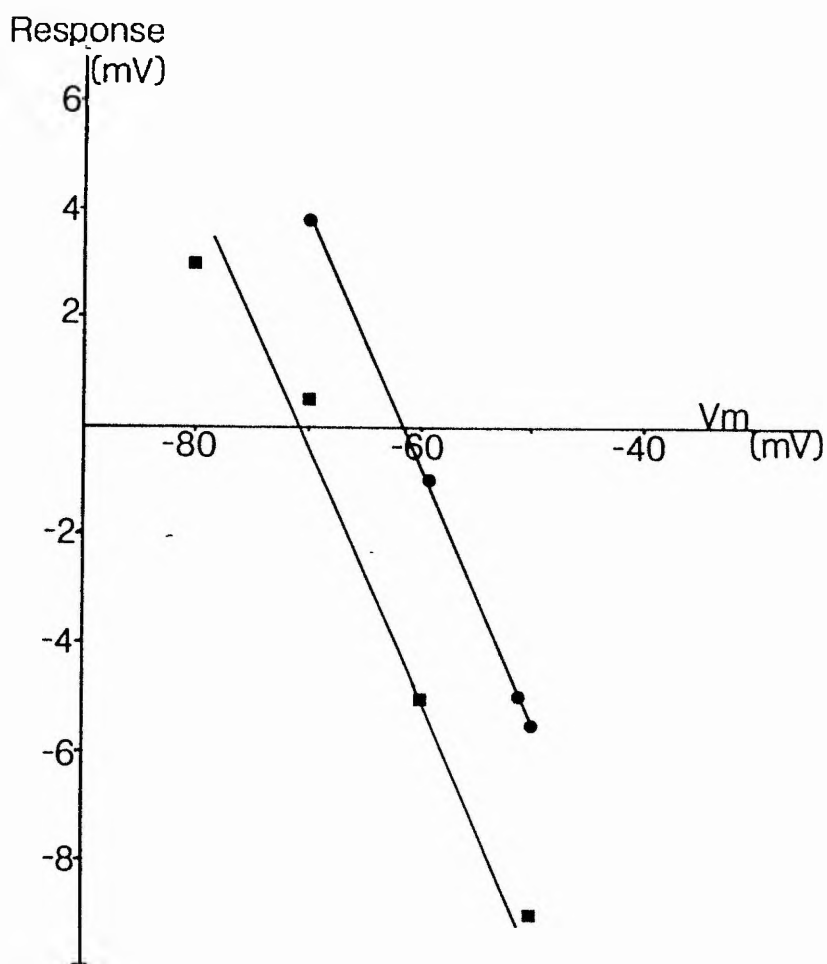
◐, represents the response in 5.8mM potassium.

◑, represents the response in 2mM potassium.

Resting membrane potential -60mV

Horizontal axis: membrane potential [mV].

Vertical axis: size [mV] and sign of the neurotensin
response.



V.7.1] RESPONSES TO NEUROTENSIN

The postsynaptic responses were hyperpolarizations associated with a decreased input resistance, fig V.30. Reversal potentials between neurones were from -70 to -45mV . The peak of the response was attained rapidly, usually within the first second, after which it lasted for between 5 to 30sec. No voltage dependence was apparent over the membrane potential range -80 to -45mV , furthermore, these responses did not show appreciable desensitization during repeated or prolonged administration. These hyperpolarizing responses were also present in recording medium which contained high magnesium/low calcium concentrations. This suggested that these responses were of a postsynaptic origin. There was the possibility of a slow developing, depolarization accompanied by an increased input resistance, [cf. Stanzione and Zieglansberger, 1983]. Such a response may have been masked by the rapid hyperpolarizing response. Therefore, the responses evoked by neurotensin were checked at more positive membrane potentials. No evidence was found for any depolarization accompanied by an increased input resistance, even at the more positive membrane potentials, fig V.30.

It was inferred from the reversal potential that the ionic species responsible for a majority of the membrane potential effects would be either chloride or potassium ions. Experiments were made with low chloride containing medium. Of 3 neurones tested, no change in reversal potential of the response was seen between the normal and low chloride recording media. Following this, recording medium which contained a low potassium concentration was used. The reversal potential was changed in the direction predicted from the Nernst

FIGURE V.33

NEUROTENSIN RESPONSE: INCREASED PRESYNAPTIC ACTIVITY

Voltage recording from a spinal neurone, details as for fig V.1. Neurotensin was applied by pressure ejection [100uM, 250msec, arrowheads]. This response was typically an apparent increase in the frequency of presynaptic activity [i.p.s.p.s]. Recordings were made in normal recording medium.

Resting membrane potential -55mV

Scale bars 10mV and 2sec.



L

FIGURE V.34

NEUROTENSIN: AMINO ACID MODULATION ?

Voltage recordings made from spinal neurones, details as for fig V.1. Neurotensin was applied by microperfusion [100uM, bar]. Microperfusion was immediately before the application of amino acid. Dose cycle of 1 per 40sec.

Upper recording: control amino acid response

Middle recording: neurotensin then the response evoked by amino acid

Lower recording: amino acid control after neurotensin

AMINO ACIDS

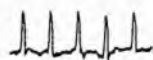
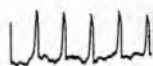
a) GABA, applied by iontophoresis [20nA, bar]. Resting membrane potential -59mV.

b) Glycine, applied by pressure ejection [50uM, 500msec, arrowheads]. Resting membrane potential -55mV.

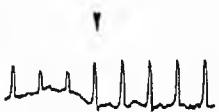
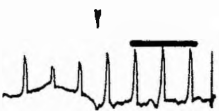
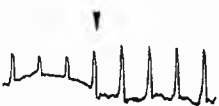
c) L-Glutamate, applied by pressure ejection [100uM, 150msec, arrowheads]. Resting membrane potential -56mV.

Scale bars for a) and b) 10mV and 2sec; located between a) and b).

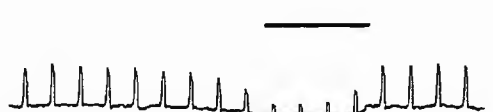
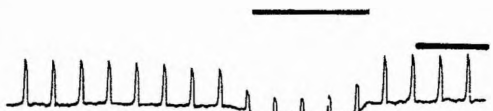
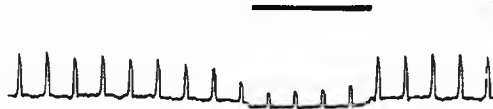
Scale bars for c) 10mV and 5sec; located between b) and c).



3



q



a

relationship, figs V.31 and V.32.

V.7.2] PRESYNAPTIC RESPONSES

On five occasions the application of neurotensin repeatedly increased the number of synaptic potentials recorded from the impaled spinal neurone, fig V.33. These effects appeared restricted to increases in i.p.s.p.s which were not desensitized after repeated application of peptide. Following this, the peptide was discretely applied to the cell layer around the responsive neurone. This study showed that these effects could not be localized to any particular nearby cell body. Furthermore these responses were predominantly found on the processes close to the perikaryon of the impaled neurone.

V.7.3] MODULATION OF AMINO ACID RESPONSES

Experiments were performed using the same protocol as for the enkephalins, cf. section III.7. No changes in size or duration of the amino acid responses were evident during neurotensin treatment, fig V.34.

FIGURE V.35

GLYCYL L-GLUTAMATE, THE FASTER RESPONSE

Voltage recordings from a spinal neurone, details as for fig V.1. Glycyl L-glutamine was applied by pressure ejection [100uM, 200msec, arrowheads]. Response shown at a range of membrane potentials [Vm]. Resting membrane potential -60mV.

a) Neurone bathed in high magnesium/ normal potassium [5.8mM] recording medium.
Reversal potential of the response -70mV.

b) Neurone bathed in high magnesium /low potassium [2mM] recording medium.
Reversal potential of the response -82mV.

Scale bars 10mV and 2sec.

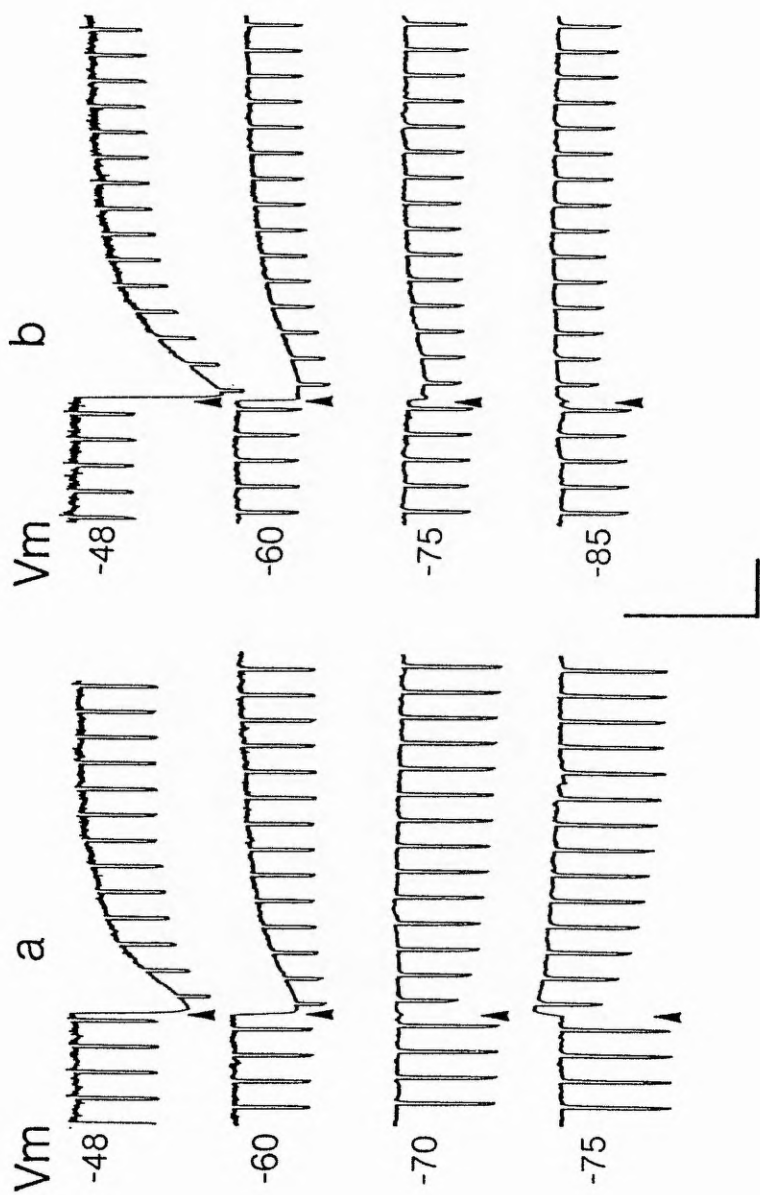


FIGURE V.36

GLYCYL L-GLUTAMINE, THE SLOWER RESPONSE:

AFFECT OF INCREASING THE INTRACELLULAR CHLORIDE CONCENTRATION

Voltage recordings from the same spinal neurone, details as for fig V.1. Glycyl L-glutamine was applied by pressure ejection [$100\mu\text{M}$, 250msec, arrowheads]. Response shown at a range of membrane potentials [Vm]. Recordings were made whilst the neurone was bathed in normal recording medium.

a) Recording electrode filled with potassium acetate [2M].

Reversal potential of the response -52mV . Resting membrane potential -55mV .

NEURONE REIMPALED

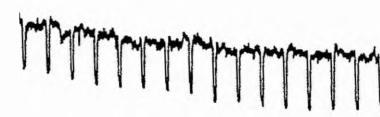
b) Recording electrode filled with potassium chloride [2M].

Reversal potential of the response extrapolated to -30mV . Resting membrane potential -52mV .

Scale bars 10mV and 2sec.

a





Vm

-45

-55

-65

b

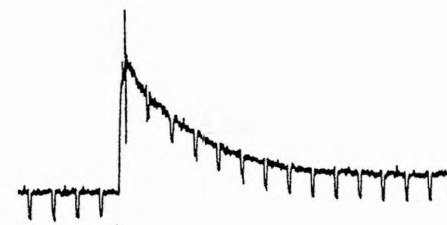
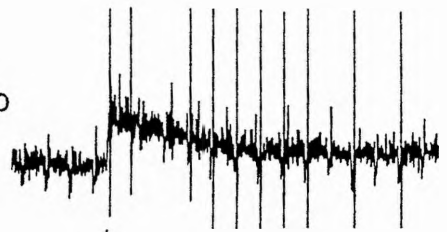


FIGURE V.37

GLYCYL L-GLUTAMINE: THE BIPHASIC, FASTER AND SLOWER, RESPONSE
AFFECT OF ALTERING EXTRACELLULAR POTASSIUM CONCENTRATION

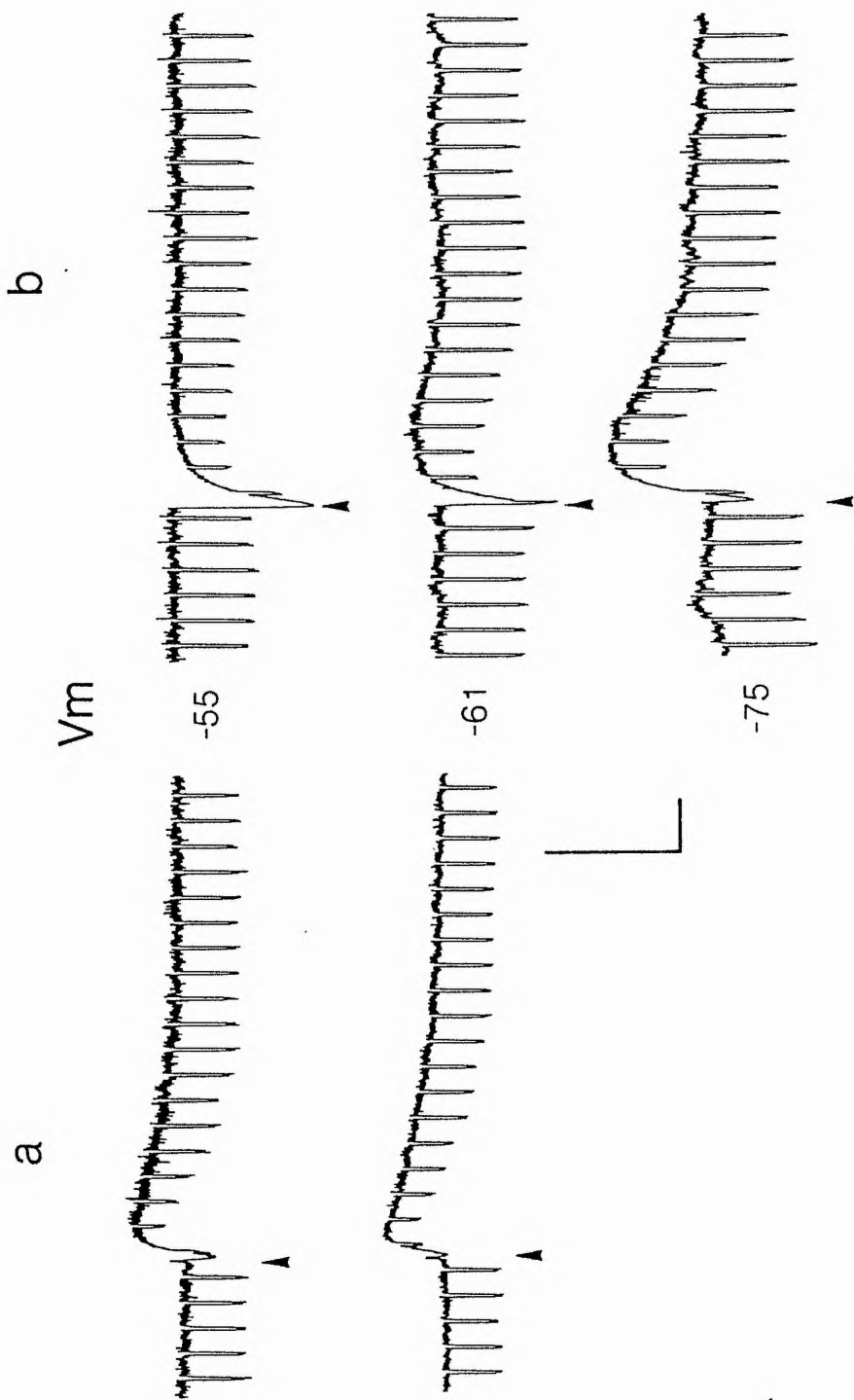
Voltage recordings made from a spinal neurone, details as for fig V.1. Glycyl L-glutamine was applied by pressure ejection [100uM, 400msec, arrowheads]. Response shown at a range of membrane potentials [Vm].

a) Neurone bathed in normal potassium [5.8mM] containing recording medium.

b) Neurone bathed in low potassium [2mM] containing recording medium.

Resting membrane potential -55mV.

Scale bars 10mV and 2sec.



V.8] GLYCYL L-GLUTAMINE

Of 81 cells tested with glycyl L-glutamine [pipette concentration of between 10 and 100uM], responses were evoked from only 15, [18% of the neurones tested with this peptide]. Two types of response were found, both of which were inhibitory. Both types of response were hyperpolarizations accompanied by a decreased input resistance. The differences between them were in duration of action and reversal potential. Usually, both of the responses were present on the same neurone which gave rise to a mixed/biphasic action, fig V.37.

V.8.1] RESPONSES TO GLYCYL L-GLUTAMINE

A] The response with the fastest onset had a reversal potential of between -75 and -60mV, fig V.35. The onset was almost instantaneous, usually reaching a maximum before the pressure pulse had ceased. When observed in isolation, this type of response slowly decayed over 15 to 30sec following the peak of the effect. No voltage dependence was seen throughout the membrane potential range studied, [-85 to -45mV].

Lowering of the extracellular potassium ion concentration altered the reversal potential of these responses. Substituting recording medium which contains a low potassium concentration [2mM potassium] for the normal recording medium [5.6mM potassium] altered the reversal potential of these responses by circa -15mV, fig V.35. The expected change would be in the range -20 to -15mV, depending upon the extracellular potassium ion concentration. Perfusion with

low chloride recording medium did not affect these responses.

B] The slower responses had reversal potentials of approximately -55mV, which was close to the resting membrane potential for these neurones, fig V.36. These actions were slower in onset and offset than those discussed above, [A]. These responses reached a maximum within 5sec of their onset and took up to 1 min to recover. No voltage dependence was seen.

The slower responses were possibly sensitive to changes in potassium ion concentration, fig V.37. However, only a small change was found when the extracellular chloride ion concentration was altered. By impaling neurones twice, firstly with an electrode containing 2M potassium acetate, and secondly with an electrode using 2M potassium chloride as the electrolyte, an involvement of chloride ions in these responses was demonstrated, fig V.36. After intracellular loading with chloride ions, the response was seen as a rapid depolarization with an extrapolated reversal potential of between -30 and -5mV.

FIGURE V.38

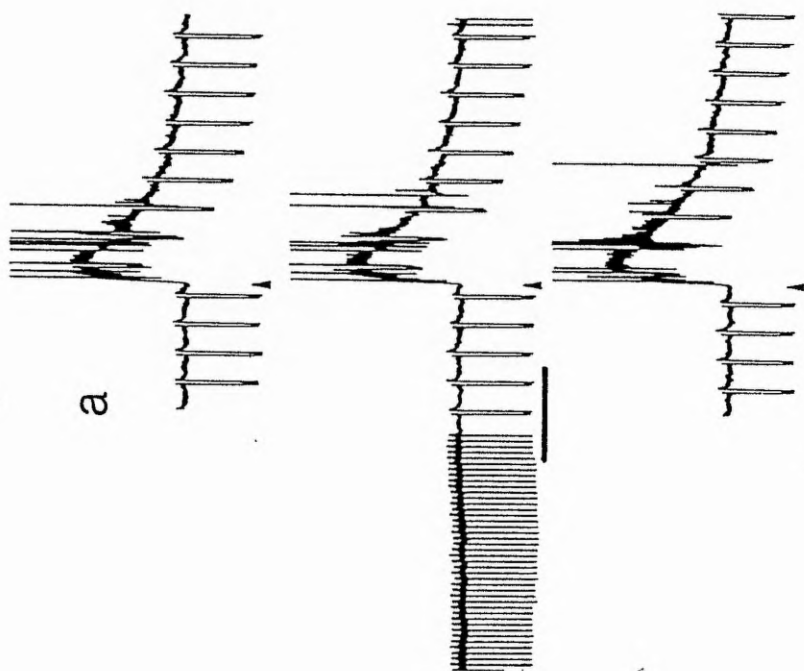
GLYCYL L-GLUTAMINE: AN L-GLUTAMATE ANTAGONIST ?

Voltage recordings from spinal neurones, details as for fig V.1. These neurones did not directly respond to glycyl L-glutamine. The peptide [100uM] was microperfused [bar] prior to applying L-glutamate. Dose cycle for L-glutamate was 1 per 30sec

Upper recording: control amino acid response. Middle recording: peptide microperfusion followed by L-glutamate application. Lower recording: control amino acid response, after peptide.

a) L-glutamate response associated with a decreased input resistance. L-glutamate applied by pressure ejection [100uM, 200msec, arrowheads]. Recording made in normal recording medium. Resting membrane potential -57mV. Scale bars, 10mV and 2sec.

b) L-glutamate response associated with an increased input resistance. L-glutamate applied by pressure ejection [50uM, 100msec, arrowheads]. Recording made in high magnesium recording medium. Resting membrane potential -60mV. Scale bars 20mV and 2sec.



a

b

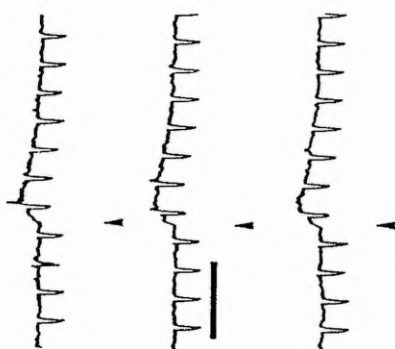


FIGURE V.39

GLYCYL L-GLUTAMINE: AMINO ACID MODULATION/ANTAGONISM ?

Voltage recordings made from spinal neurones, details as for fig V.1. As with the other peptides, neurones which showed no obvious electrical response to glycyl L-glutamine were used.

Glycyl L-glutamine [100uM] was applied by pressure ejection prior to a dose of amino acid.

Top recording: control response to the amino acid.

Middle recording: pressure ejection of peptide prior to amino acid application.

Bottom recording: control response to amino acid, after peptide.

a) GABA application by iontophoresis, [15nA]. Resting membrane potential -54mV.

b) Glycine application by pressure ejection, [100uM, 200msec]. Resting membrane potential -58mV.

Scale bars 10mV and 2sec.

a



b



V.8.2] MODULATION OF AMINO ACID RESPONSES

Glycyl L-glutamine was tested for modulatory activity on the GABA and glycine responses. The same protocol was followed as in the other peptide modulation experiments, cf. section III.7. No attenuation of the amino acid responses was observed in the presence of glycyl L-glutamine, fig V.3 .

V.8.3] GLYCYL L-GLUTAMINE AS A POSSIBLE ANTAGONIST OF L-GLUTAMATE

One of the original reasons for obtaining glycyl L-glutamine was the similarity between it and the L-glutamate analogues/antagonists, gamma-D-glutamyl glycine, [cf. Davies and Watkins, 1981; Collingridge, Crunelli, Forda and Kelly, 1982] and glutamyl diethyl ester [Watkins and Evans, 1981]. A similar protocol was used as for the amino acid/enkephalin modulation experiments, cf. section III.7. The concentrations of glycyl L-glutamine used were those which evoked responses from 18% of the spinal neurones. However, no antagonism/modulation of either L-glutamate-evoked response was found, fig V.3 .

CHAPTER VI

DISCUSSION

PEPTIDE RESPONSES FROM THE SPINAL NEURONES IN CULTURE

This thesis has described a series of responses which were evoked from neurones in a dissociated cell culture of spinal cords from the mouse embryo. The emphasis of this chapter will be on the responses evoked from those neurones by the following peptides: met⁵- and leu⁵- enkephalins, FMRFamide, neurotensin and glycyl L-glutamine.

VI.1] NEURONAL RESPONSES TO ENKEPHALINS

No conclusive evidence was obtained concerning the ionic mechanisms which underlie the enkephalin responses. The major reasons for this were: the inability to manipulate the extracellular ion concentration at the start of the project because of the initial emphasis on morphological and electrophysiological studies, and the low number of neurones subsequently found capable of responding to the peptides.

VI.1.1] INHIBITORY RESPONSES

A common response of the neurones to enkephalin was a hyperpolarization associated with a decreased input resistance. This effect was obtained from approximately 8% of the neurones tested with enkephalin: the equivalent of over 42% of those neurones which responded to enkephalin. Reversal potentials associated with this response varied from -70 to -40mV, between the individual neurones. The ionic mechanism[s] underlying this response were not studied. However, from the range of reversal potentials obtained, it appeared that a change in the conductance of more than one ionic species was involved.

A similar hyperpolarizing response from cultured mouse spinal neurones was described by Barker and co-workers, [1978a]. These workers suggested the presence of an enkephalin response which was mediated by chloride ions. This type of response was found in 30% of their cultured neurones. Barker and co-workers, [1980a] reported that this response did not desensitize on repeated application of peptide. However, such large variations in reversal potential between neurones was not reported by Barker and co-workers [1978a;1980a].

Neurones in the rat locus coeruleus gave responses to enkephalin which corresponded with an increase in a potassium conductance, [Williams et al., 1982]. These responses appeared as hyperpolarizations accompanied by a decrease in the input resistance. However, unlike the responses described in this thesis and those described by Barker and co-workers [1978a;1980a], these hyperpolarizations were reported to be voltage dependent. A hyperpolarizing response with a similar appearance was evoked by enkephalin from dorsal horn neurones in the spinal cord slice [Murase, Nedeljkov and Randic, 1982; Yoshimura and North, 1983]. These responses were shown to be mediated by an increase in the membrane conductance to potassium ions. The involvement of potassium usually means that the equilibrium potential for the response would be more negative than resting membrane potential, by over -30mV [Williams et al., 1982; Yoshimura and North, 1983]. This appears to preclude the responses which were evoked by enkephalin from these cultured neurones. However, the responses evoked by neurotensin from cultured neurones in this project [McCarthy, 1984] were largely mediated by an increased potassium conductance. These neurotensin

responses had reversal potentials over the range -70 to -45mV. This is similar to the range of reversal potentials recorded for this type of enkephalin response. Even so, the possibility of a large chloride component, as suggested by Barker et al., [1978a;1980a] should not be disregarded.

It is obvious that further studies [such as by manipulating the concentration of ions either intra- or extra-cellularly and by using more sophisticated recording techniques] will be the only way of resolving the question of the underlying mechanism of this response. At the same time, other possibilities for the underlying mechanism to this response must also be considered. These alternatives must include increases in the membrane permeability to mixtures of ions; e.g., both a chloride conductance and a potassium conductance. However, the possible involvement of a sodium conductance [as with responses to FMRFamide, McCarthy and Cottrell, 1984] or an underlying calcium conductance [Owen, Segal and Barker, 1984] cannot be overlooked.

On 4 occasions a slowly developing inhibitory response was seen after leu⁵-enkephalin application. These responses were manifested as a slight hyperpolarization with a very small decrease in input resistance. On the same neurones, met⁵-enkephalin evoked a hyperpolarization associated with a large decrease in membrane resistance. The latter responses were of a rapid onset and had reversal potentials close to the resting membrane potential of the neurone. Both of these response types reduced the rate of action potential firing of the impaled neurone. However, the met⁵-enkephalin effect was apparently the most potent. Before each

series of experiments the opioid peptides were freshly prepared at similar concentrations. Even so, larger pressure pulses and periods of application were required to evoke responses with leu⁵-enkephalin compared with those required for met⁵-enkephalin. Thus, leu⁵-enkephalin appeared less potent at evoking responses from these neurones than the met⁵-enkephalin. The responses evoked by leu⁵-enkephalin also took much longer to reach a maximum and subsequently longer to recover than did those of the equivalent met⁵-enkephalin response. However, both of these responses were dose-dependent. When the met⁵-enkephalin was applied during a leu⁵-enkephalin response the effect evoked by the former peptide was attenuated. This effect argues for a partial-agonist activity for enkephalin, assuming that one is satisfied that similar concentrations of "neuro-active" enkephalin were reaching the receptors and that the same receptors were being activated.

It has been well documented that met⁵-enkephalin is more potent than leu⁵-enkephalin at the Mu, Delta and Kappa sub-types of opioid receptor [Kosterlitz, Magnan, Paterson, 1982; Waterfield, Smokcum, Hughes, Kosterlitz and Henderson, 1977]. It has also been shown that potent opiate agonists at one receptor sub-type can act as antagonists in a tissue containing a predominance of another receptor sub-type, [Henderson, Robinson and Sim, 1982; Huidobro, Huidobro-Toro and Miranda, 1980]. It therefore came as no surprise that given differing mixtures of receptors [cf. Werz and MacDonald, 1984], either one or both of these opioid peptides may evoke a response from a neurone. This was further illustrated by the number of neurones found in this project which only responded to one of these opioid peptides. Any partial agonist activity related to

enkephalins may not be of much physiological importance because of their short half lives in vivo [Hambrook, Morgan, Rance and Smith, 1976; Dupont, Cusan, Garon, Alvarado-urbina and Labrie, 1977]. However, this may have a functional significance to opioid peptides which have a lower susceptibility to enzymatic degradation, e.g., B-endorphin [Chang, Rao and Li, 1978].

VI.1.2] EXCITATORY RESPONSES

Further responses evoked from the cultured neurones by enkephalin were accompanied by a decrease in input resistance. These effects were manifested as depolarizations and were found in approximately 7% of impalements. No voltage dependence was found over the range of potentials available. The reversal potentials of these depolarizations were extrapolated to circa -20mV. Although the responses seen here were of a superficially similar nature to those depolarizations reported by Barker and co-workers [1980a], a more detailed investigation revealed that they were in fact subtly different. The depolarizations reported here differed in their slower time course and their lack of desensitization during repeated application. The reversal potential of the enkephalin-evoked depolarizations described by Barker and co-workers varied from -4mV to +20mV, over three reports [Barker et al., 1978a; 1980a; 1980b]. These are more positive values than that extrapolated for the enkephalin evoked depolarizations described in this thesis. However, this may have been due to the inaccuracies incurred in the extrapolation. This type of depolarization may be compared with responses evoked by L-glutamate and DL-kainate from cultures of mouse spinal neurones, [MacDonald and Wojtowicz, 1980; fig V.1 and V.3].

The major differences between these responses were the smaller change in input resistance of the enkephalin response and the slower recovery. The depolarizing responses evoked by both enkephalin and L-glutamate transiently increased the rate of action potential firing of the impaled neurone. This effect was more noticable with the amino acid, possibly because of an underlying "NMDA-type" response.

The type of depolarizing response found in this project had extrapolated reversal potentials which were not close to that of any single ionic species. This indicated that more than one species of ion, and/or an undetected voltage dependence may underlie these responses. A further possibility was suggested by Marshall and Engberg, [1979] to explain the action of noradrenaline on spinal motoneurons of the cat; namely the activation of a non-selective cation channel which would allow a mixed flow of sodium and potassium ions through the cell membrane. This could produce a reversal potential which would fall between those of the ions concerned, [cf. Takeuchi and Takeuchi, 1960]. The scarcity of neurones giving this type of response coupled with the inability to reliably change the membrane potential outside the range -80 to -40mV, made study of this hypothesis extremely difficult within the time available.

In other preparations relatively few responses evoked by enkephalins have shown any similarity to these depolarizations. Using extracellular recording techniques, these responses would appear as excitations with a rapid onset and offset, relative to those produced by depolarizations associated with an increased input resistance. Rapid excitations were reported when opioids were administered to feline Renshaw cells and rat cerebral cortical cells

in vivo [Davies and Dray, 1978]. These effects were apparently not desensitized by repeated application but were abolished by naloxone.

In intracellular studies, Barker and co-workers, [1978a] described a depolarizing response which was accompanied by little or no increase in input resistance. These responses preceded abrupt depolarizing events. Such events were reported as abrupt changes in the membrane potential and were sufficient to make analysis of other responses difficult, if not impossible. As a consequence only the initial part of these responses was described by Barker et al., [1978a]. The details which are available compare favourably with those responses evoked by the enkephalins in this project. These responses were voltage dependent depolarizations associated with an increase in input resistance [Cottrell and McCarthy, 1982]. The accompanying change in input resistance was usually quite small at resting membrane potential and therefore could have been missed quite easily. As a precaution, the impaled neurone was depolarized prior to application of peptide. This ensured that voltage dependent responses of this kind could be observed. Even with this precaution, this type of response was evoked by enkephalin from only 2% of the cultured spinal neurones which were tested. No desensitization of these responses was seen with repeated or prolonged application of peptide.

These responses were capable of causing a potent and long-lasting increase in the rate of action potential firing; on some occasions even initiating the generation of action potentials from previously quiescent neurones. Such a long lived excitation would be distinctive even in an extracellular study of neuronal

activity. Long-lasting excitations of neuronal activity were described by Stone, [1983] in studies of the cerebral cortex of the rat, in vivo. 50% of tests with enkephalin produced the prolonged excitation, on occasion lasting for up to 2min.

Another possible site of action for these responses was the rat hippocampus. Lynch and co-workers, [1981] suggested that the opioid peptides may be acting through an increased coupling of the dendrite to a spike initiation area on the pyramidal cells. An increased input resistance of the dendritic/somatic membrane, may have mediated such a change in coupling. However, these workers were unable to corroborate this as they only studied the rat hippocampus at an extracellular level. Intracellular recordings from the soma of pyramidal cells [Masukawa and Prince, 1982] have not found any evidence for such a mechanism. They studied these responses at the cell body and therefore, it is still possible that a dendritic response of the type envisaged from the report of Lynch et al., [1981] may exist, as yet undetected in the dendrites.

Similar depolarizing responses have been evoked from cultured spinal neurones by other putative neurotransmitters. Green and Cottrell, [1983] found only a few of their cultured spinal neurones which responded to 5-hydroxytryptamine, [5-HT].

One type of response was a depolarization accompanied by a increase in input resistance. These responses were also voltage dependent. 5-HT has also evoked voltage dependent depolarizations from neurones in intact tissue; facial motoneurones of the rat [Vandermalen and Aghajanian, 1980] and identified neurones of both the snail [Cottrell, 1982] and Aplysia [Pellmar, 1984]. Some of

these responses appeared to be mediated by the reduction of a potassium ion conductance [Cottrell, 1982; but cf. Pellmar, 1984]. However, the primary involvement of a calcium ion conductance or some other factor [e.g., cAMP] may, or may not be incidental in the operation of such a mechanism, [Pellmar 1984; Barnes and Dunbar personal communication, 1985]. The results of an intracellular study by Nowak and MacDonald [1982] described similar depolarizing responses following substance P application to cultured spinal neurones. A decreased potassium conductance was also suggested as the mechanism underlying these responses. FMRFamide evoked similar depolarizations from the cultured neurones used in this project, [McCarthy and Cottrell, 1984]. However, as with the responses to substance P, those responses evoked by FMRFamide from the cultured neurones were not voltage dependent. A similar voltage dependent response was however reported by MacDonald and Wojtowicz [1982]. These responses evoked from cultured neurones by NMDA suggested a further [although tenuous] possibility for an underlying mechanism to the peptide-evoked voltage-dependent depolarizations. In these responses the increased input resistance was apparently caused by a blockade of ion channels which had been opened by NMDA. It was shown that magnesium ions blocked these NMDA-opened ion channels [Mayer, Westbrook and Guthrie, 1984]. An apparent voltage dependence of this response was also a feature of the channel blockade. At resting membrane potentials [-60 to -50mV] the blockade of the channels was strong, whereas at more positive potentials the blockade became less severe, being almost non-existent at +20mV.

VI.2] NEURONAL RESPONSES TO FMRFamide

FMRFamide responses were evoked from approximately 19% of the cultured spinal neurones which were tested. Two distinct types of direct/postsynaptic FMRFamide responses were found. Both of these effects were evoked simultaneously from approximately 30% of those neurones which responded to FMRFamide.

VI.2.1] INHIBITORY RESPONSES

Half of the responses evoked by FMRFamide from these cultured neurones were accompanied by a decreased input resistance. These responses were either hyperpolarizing or depolarizing, the sign being dependent upon both the reversal potential of the response and the membrane potential of the neurone. Voltage dependence was not apparent in these responses, over the range of membrane potentials -90 to -40mV. Repeated or prolonged application of peptide did not cause desensitization of these effects. A major difference between these responses and others evoked from the cultured spinal neurones was the large variation in reversal potential between neurones. This variation in reversal potential ranged from -70 to -25mV and suggested that these responses were composites of changes in membrane permeability to more than one ionic species. Evidence presented in this thesis supported this and indicated that the ions involved were chloride and sodium. Apparently, potassium ions had no measurable involvement. This was in contrast with the report by Cottrell [1982; et al. 1984] who showed that an increased potassium ion conductance was involved in FMRFamide responses with a similar appearance which had been evoked from identified snail neurones. The

difference in ionic mechanism may possibly be due to species differences.

VI.2.2] EXCITATORY RESPONSES

The other half of the cultured spinal neurones responding to FMRFamide did so with a depolarization associated with an increase in input resistance. These responses were apparently not voltage dependent, unlike the similar ones evoked by enkephalin from these neurones, Cottrell and McCarthy [1982]. The reversal potential of these depolarizations was affected by a change in the extracellular potassium ion concentration, thus, suggesting an involvement of potassium ions. In this case, the mechanism underlying these effects would most likely have been a reduction in the membrane permeability to potassium ions. Similar responses have been reported in tests with FMRFamide on identified neurones of the snail, *Helix aspersa* [Cottrell, 1982; Cottrell et al., 1984; Paupardin-Tritsh, Colombaioni, Vidal and Gerschenfeld, 1985]. However, those responses were mainly voltage dependent, unlike their counterparts from cultured spinal neurones [McCarthy and Cottrell, 1984]. The voltage dependence was apparently linked to an interaction of the affected potassium conductance with either a calcium conductance [Cottrell et al., 1984; Paupardin-Tritsh et al., 1985] or a second messenger system, cAMP [Paupardin-Tritsh et al., 1985]. As a further possibility, the potassium channel acted upon by the peptide may itself have been voltage dependent.

Substance P responses evoked from similarly cultured mouse spinal neurones were also depolarizations accompanied by an increase

in input resistance, [Nowak and MacDonald, 1982; Green, personal communication]. These responses evoked by substance P were also affected by changes in the extracellular potassium ion concentration. However, the lack of voltage dependence of these FMRFamide and substance P responses distinguished them from the similar, and voltage dependent responses evoked by 5-HT and enkephalin from cultured spinal neurones [Green and Cottrell, 1983; Cottrell and McCarthy, 1982], as well as those depolarizations of identified molluscan neurones evoked by FMRFamide [Cottrell, 1982; Cottrell et al., 1984; Paupardin-Tritsh et al. 1985].

VI.2.3] BIPHASIC RESPONSES

The occurrence of both types of FMRFamide response simultaneously from the same neurone complicated the analysis of the individual responses. The characteristics of these multiple [biphasic] responses varied between neurones. Biphasic responses to FMRFamide have also been described elsewhere. Cottrell [1982] described the response of an identified snail neurone to FMRFamide. The response was composed of two effects; a reduction in a potassium conductance and an increase in a potassium conductance, the former being voltage dependent. Attempts were made to locate "hot-spots" or areas which preferentially gave one or other of the FMRFamide responses. However, these studies proved inconclusive.

VI.2.4] ENKEPHALIN AND FMRFamide RESPONSES FROM THE SAME NEURONE

Neurones gave different responses to separately applied met⁵-enkephalin and FMRFamide. In these experiments, only 2 neurones, out

of 22 tested, responded to both of these peptides. Each of the peptides evoked a depolarization from those 2 neurones. The type of response evoked by each peptide appeared to be mediated by a different ionic mechanism. An increase in input resistance was associated with the FMRFamide responses and a reduction of input resistance was observed during the actions evoked by met⁵-enkephalin from these neurones. This suggested that FMRFamide was not acting through the same receptor/ion conductance complex as met⁵-enkephalin, confirming at an intracellular level the study by Gayton [1982].

VI.3] NEURONAL RESPONSES TO NEUROTENSIN

Only 5 of the 201 cultured spinal neurones which were tested with this peptide gave a postsynaptic response. All of the direct/postsynaptic effects of neurotensin were found in one preparation. A further 8 cells which did not respond to neurotensin were also found in this preparation; 7 before and 1 after the 5 neurones which gave the responses.

At resting membrane potential, all of the neurotensin responses were hyperpolarizations associated with a decreased input resistance. These neurotensin responses also caused an apparent reduction in any spontaneous activity recorded from neurones. A further characteristic of these responses was the lack of voltage dependence over the membrane potential range -85 to -40mV. Repeated or prolonged application of peptide caused little or no desensitization of the response.

The reversal potentials of these neurotensin effects varied between neurones, over the range -70 to -45mV . This suggested an involvement with either chloride, potassium ion or, some mixture of ion species. The possible role of chloride ions was tested first. No change in reversal potential of the response was found when the extracellular medium was exchanged for one which contained a low chloride ion concentration [isethionate substituted for the chloride]. In the presence of a low extracellular potassium ion concentration [sodium exchanged for potassium] the reversal potential behaved as expected from the Nernst relationship. It may be inferred from these results that potassium ions, and not chloride ions, were responsible for a large part of this response. The presence of these responses in medium containing high magnesium and low calcium ion concentrations suggested they were of a postsynaptic origin.

Previous studies of the effects of neurotensin on neuronal activity have reported inhibitions of activity with a rapid onset and offset, [Young *et al.*, 1978; McCarthy *et al.*, 1977]. However, in a later study, Marwaha and co-workers [1980] demonstrated that the inhibitory action of this peptide on cerebellar Purkinje cells was indirect. When chemical [dopaminergic] neurotransmission was prevented, by extracellular magnesium ions, haloperidol or pretreatment of the animal with 6-hydroxydopamine, neurotensin produced an increase in neuronal activity from the cerebellar Purkinje cells. This increase in activity had a slow onset. Studies of neurotensin effects in the spinal cord have shown a predomination of excitatory actions, [Miletic and Randic, 1979]. A further study of the spinal cord, by Henry [1982], described inhibitions of neuronal activity as well as stronger excitations which were evoked

by neurotensin. It was claimed that the variations between these two studies were due to differences in sample sizes [Henry, 1982]. The excitatory responses reported by Miletic and Randic [1979] were smaller and less effective at increasing neuronal activity than those described by Henry [1982]. These differences suggested anomalies in the quality and/or area of drug application and species differences rather than purely differences in sample size as claimed by Henry [1982]. Neither of these workers reported the sensitivity of their responses to compounds which prevent chemical synaptic transmission, [e.g. magnesium ions, TTX].

The only "direct/postsynaptic" responses evoked by neurotensin which have been reported were slow excitations of neuronal activity; rat cerebellar Purkinje neurones [Marwaha et al., 1980] and periaqueductal grey neurones [Behbehani and Pert, 1984]. These excitations were long-lived with a slow onset, suggesting they could have been mediated by effects similar to those described for enkephalins and FMRFamide in this thesis; depolarizations associated with an increased input resistance. Apparent confirmation of this possibility was found in an intracellular study of neurotensin in the spinal cord, by Stanzione and Zieglgansberger [1983]. These workers have shown that dorsal horn neurones give depolarizing responses accompanied with increases in input resistance when challenged with neurotensin. However, the change in input resistance was reported to lag behind the depolarization by a few seconds and prolonged application of neurotensin caused depolarizations from which the cell did not recover. The effects of the depolarization on prolonged application of peptide could have resulted from an artefact of drug application. Similar types of response evoked by other peptides

alter input resistance by comparable amounts [150%]. However, the membrane potential change at such negative potentials [-70mV] was usually quite restricted, in the order of 10mV. The conclusions drawn from this could be that, either the response had a different underlying mechanism to the similar responses evoked by FMRFamide, enkephalins, substance P, 5-HT and even NMDA or that a part of those neurotensin responses was artefactual. Studies of the neurotensin responses of neuroblastoma X glioma hybrid cells [NG108-15] by Higashida [1984] showed a biphasic response. The initial phase was a short-lived hyperpolarization accompanied by a decrease in input resistance. These responses showed a similarity to the neurotensin responses evoked from these cultured spinal neurones. However, in the neuroblastoma the hyperpolarizing phase faded quickly, revealing a slower onset depolarization associated with little or no increase in input resistance. This latter phase resembled the description of the neurotensin response reported by Stanzione and Zieglansberger [1983], except, the responses evoked from the neuroblastoma were much smaller.

There were no measurable depolarizing responses found following the hyperpolarizations evoked by neurotensin from the cultured spinal neurones. However, it was possible that the depolarizing responses to neurotensin were voltage dependent, as were the similar responses which had been evoked by enkephalin [Cottrell and McCarthy, 1982] or 5-HT [Green and Cottrell, 1983] from these cultured spinal neurones. This suggested the possibility of depolarizing responses being masked at resting membrane potentials. To ensure against this, the cultured spinal neurones were depolarized to circa -40mV prior to the neurotensin application. Even with this precaution, no depolarizing

responses were found which had been evoked by neurotensin application.

It was possible that the rapid hyperpolarizing response would be seen only in experiments where a method of rapidly applying the neurotensin was used, [e.g., pressure ejection; Higashida, personal communication]. Evidence in support of this was found in the report by Marwaha and co-workers [1980]. These workers described how the pressure ejected peptide gave a potent depression of neuronal activity whereas the iontophoretically applied peptide evoked a lesser depression of neuronal activity.

Occasionally, increases in presynaptic activity were found which were associated with neurotensin application. These effects were repeatable and did not desensitize with frequent application. The source of these responses was not localized to any neighbouring cell body within 100um of the impaled neurone. It is a possibility that these responses were manifestations of an excitatory response evoked by neurotensin. This action could possibly have been located on nerve terminals or neuronal fibres presynaptic to the impaled neurone, or even on the postsynaptic membrane in the dendrites [cf. Lynch et al., 1981].

VI.4] NEURONAL RESPONSES TO GLYCYL-L-GLUTAMINE

Two aspects of the pharmacology of this peptide were studied. Both were related to the reported inhibition of neuronal activity following application to neurones in the rat brainstem, [Parish et al., 1983]. The first came from the structural analogy between

glycyl L-glutamine and certain L-glutamate antagonists; gamma-D-glutamyl glycine and glutamic acid diethyl ester [Davies and Watkins, 1981; Watkins and Evans, 1981]. The second was the possibility that this peptide had direct inhibitory activity when applied to the cultured neurones. As most of the cultured spinal neurones responded to the application of L-glutamate and the analogues NMDA and DL-kainate [Macdonald and Wojtowicz, 1980; this thesis] as well as numerous peptides, these neurones appeared perfect to test both possibilities.

The results from these cultured spinal neurones showed that glycyl L-glutamine did not attenuate the responses evoked by either L-glutamate, NMDA or DL-kainate. However, during one of these experiments a direct "inhibitory" activity was seen during the application of this dipeptide. On further study, approximately 20% of the neurones responded to the application of glycyl L-glutamine. Occasionally, this response was biphasic. However, both responses [phases] were hyperpolarizations accompanied by a decreased input resistance. The 2 phases differed in both time course and reversal potential. The most rapid response appeared to be mediated by an increased membrane permeability to potassium ions. The slower response appeared to have some relationship with chloride ions. However, neither response appeared to be altered by changes in extracellular chloride ion concentration [isethionate substitution for chloride]. On the other hand, after an intracellular injection of chloride ions the responses became depolarizations accompanied by a decrease in input resistance. These experiments cast doubt on data obtained using medium containing a low chloride ion concentration. Therefore, responses which were known to be chloride ion dependent

[GABA or glycine] were further tested with the same solutions. As would be expected, the reversal potential of the response evoked by either amino acid became more positive in the presence of low extracellular concentrations of chloride ions. These results suggested that glycyl L-glutamine responses were sensitive to changes of intracellular chloride ion concentrations and were relatively insensitive to changes of the extracellular chloride ion concentration. The interference of other ion species with this response, e.g., intracellular acetate or extracellular sodium, have yet to be tested. There still remains the possibility that a voltage and/or calcium sensitive chloride ion conductance or even some ion pump was involved in the slower response. Studies into mechanisms such as these would be best undertaken using more reliable methods of voltage manipulation, e.g., single/double electrode voltage-clamp or whole cell clamp.

These responses to glycyl L-glutamine resemble the 2 forms of inhibition described by Parish and co-workers [1983]. These workers reported both "fast and slow onset inhibitory actions" which were occasionally found on the same neurone. Their responses were unaffected by both naloxone and bicuculline. In this study, only naloxone was tested and found ineffective at a concentration of approximately 20uM.

Breakdown of glycyl L-glutamine into the constituent amino acids [L-glutamine and glycine], was ruled out in this thesis by the type of responses observed and an amino acid chromatography of the peptide [Smith, 1969]. Neither L-glutamine or glycine evoked a hyperpolarization associated with an increase in a potassium

conductance and the part of the response possibly mediated by chloride did not behave in the same way as that of the glycine response. Further to this, no appreciable quantity of breakdown products were found in the chromatography, especially with respect to free glycine. However, further studies should include use of the glycine antagonist strychnine. Parish et al. [1983] also produced chromatographic evidence in support of the lack of breakdown even after weeks in solution at room temperature.

It would appear from these experiments that the dipeptide glycyl-L-glutamine [or a similar peptide] may have a neurotransmitter/modulator role in the mammalian CNS at the level of the brainstem and spinal cord.

VI.5] WHY DID SO FEW NEURONES RESPOND TO PEPTIDE?

It was reported that on average these peptides evoked responses from less than 18% of the cultured spinal neurones. This phenomenon was not exclusive to the cultures maintained for use in this project. Other groups have reported that only small percentages of cultured spinal neurones responded to either substance P [Nowak and MacDonald, 1982; Barker et al., 1980a], 5-HT [Green and Cottrell, 1983] or enkephalins [Barker et al., 1980b].

One should expect that only a small percentage of the cultured neurones, from a dissociated culture of a whole tissue, would give responses when challenged with the peptides. This is because the total number of cells which are capable of responding to a peptide

tends to be "diluted" in such a culture. Indirect evidence of this came from the study by Neale, Barker, Uhl and Snyder [1978]. These workers described how only 1 to 3% of the cells in their primary dissociated cell cultures of mouse spinal cords contained enkephalin-like immunoreactivity. Their results showed the extent of the relative dilution of these neurones in dissociated culture. From these results one may obtain some indication of the dilution, which could be expected, of those neurones capable of responding to opioids or any other peptides.

Even in situ the percentage of neurones which have responded to a peptide has seldom been greater than 70% of those tested. The results of an intracellular study of neurones from Lamina II of the rat spinal cord by Yoshimura and North [1983] illustrated this. They reported that responses were evoked from only 13 neurones of 26 which were tested with opioid. In certain brain regions the percentage of neurones which gave responses to enkephalin was higher; e.g., the rat hippocampus where 136 neurones from 150 responded to enkephalin, [Zieglgansberger et al., 1979]. In the locus coeruleus, most of the neurones which were tested with normorphine or DADLE gave a response, [North and Williams, 1983a].

During the preparation of spinal cord cultures for use in this project no attempt was made to isolate or selectively remove areas of tissue [e.g. dorsal horn] which may have contained neurones capable of responding to peptides. Therefore, one would expect that only a small percentage of the cultured neurones could respond to peptide. The small percentage of neurones which responded to peptides in this project prompted a study of individual cell morphology. The

objective was to discover if there were any distinguishing features related to these neurones. A variety of morphologically distinct neurones were found [the DRG neurones]. However, the study proved inconclusive with respect to responses of the neurones to the peptides which could be of, apparently, any shape or size.

The neurones which responded to peptides were often found in small clumps or groups. More often than not, if one neurone responded to a peptide most, if not all, of the spinal neurones present in that group of neurones [up to 5] would also respond to that peptide. The responses obtained from all of the neurones in any group were usually similar. A prime example was that of the neurotensin responses described in this thesis. Those neurones which could respond to peptide were usually situated either amongst a larger collection of cells or, be isolated from other neuronal cell types. The individual neurones within these groups usually had various soma diameters and different morphologies. This variation did not allow the location of the responsive neurones to be determined by visual study alone.

There were a variety of possible explanations for these observations. Firstly, there may have been some correlation between the cells containing peptide and those which responded to peptide application. A further possibility was that all of the group of responding neurones were derived from the same cell. This assumes that some of the neurones taken from the embryo were at the blast stage of their development, i.e. not fully differentiated [cf. Kriegstein and Dichter, 1984]. Evidence from immunohistochemical studies of peptides in cultured neurones supported this proposal

[Neale et al., 1978]. These studies showed that a reasonable percentage of cells which contained the immunoreactive material were found in groups or clusters. A third possibility relies on the ability of the neurones to migrate in the culture dish, [O'Lague, Potter and Furshpan, 1978]. This suggests that similar neurones migrate towards each other, presumably along some chemical gradient, thus, leading to a grouping together of "like" cells which would be especially noticable in older cultures.

The percentage of neurones which were found capable of responding to peptide varied between culture preparations. A greater variation was apparent between batches of cultures over months of study than between culture dishes from individual batches. At the start of the project the percentage of neurones which responded to enkephalin was approximately 50% of those tested. Only 6 months after the first responses to enkephalin had been found the percentage of neurones responding to these opioids had declined to $\leq 5\%$. FMRFamide was introduced into the project as the percentage of neurones responding to enkephalins was decreasing. The number of neurones which responded to FMRFamide during the start of the study formed only $\leq 10\%$. After 6 months this had increased to 40% followed by a decrease to $\leq 5\%$ after a further 2 months. All of the direct/postsynaptic responses evoked by neurotensin were found in one preparation.

This variability was apparent, if not stated, in other studies of cultured neurones. Barker et al. [1978a] reported that only 30% of their cultured spinal neurones gave a postsynaptic "neurotransmitter-like" response to the enkephalins. In a later

study by the same group [Barker et al., 1980a], they reported that only circa 10% of the cultured neurones which were tested gave a response to either enkephalin or substance P. In the latter report, the authors also described a large degree of variation in the number of neurones responding to enkephalin between the different batches of spinal cord cultures. Werz and MacDonald [1984] described the variability in distribution of opioid receptor sub-types between DRG's in batches of cultured spinal neurones.

The variations between batches of cultures were attributed to possible changes in tissue/cellular contaminants which had been unwittingly added to the culture during the preparation. In the testing of this hypothesis, and if possible to discover the nature of the contaminant, a series of extremely careful dissections were undertaken. Known tissues, other than spinal cord, were added to a proportion of the spinal cords from one group of embryos and a dissociated culture prepared from this mixture. Cultures of these mixtures were prepared and maintained using the same protocol as used with the spinal cords. A series of intracellular studies were undertaken with neurones from these mixed cultures to ascertain whether a greater percentage of those neurones responded to the peptides. The results of this study showed that the mixed cultures did not contain a higher percentage of neurones which were capable of responding to peptide than those from the control/pure spinal cord cultures.

VI.6] AMINO ACID PHARMACOLOGY OF THE SPINAL NEURONES

A preliminary study was performed using excitatory and

inhibitory amino acids. There were two main reasons for undertaking this study. The first was to facilitate the development of skills pertaining to the project; such as the application of drug containing solutions onto neurones during an intracellular recording. The second reason was to compare the results obtained with previous studies.

VI.6.1] EXCITATORY AMINO ACIDS

L-Glutamate and its analogues DL-kainate and NMDA, evoked depolarizing responses from the cultured neurones. The L-glutamate responses were predominantly depolarizations accompanied by a decrease in input resistance. These were similar to those reported by Ransom and co-workers, [1977b] and MacDonald and Wojtowicz [1982]. DL-Kainate evoked a similar type of response from these neurones to that produced by L-glutamate [MacDonald and Wojtowicz, 1982]. Both of these responses had extrapolated reversal potentials of approximately -20mV . The NMDA responses evoked from these neurones were depolarizations associated with increases of the input resistance. Mayer and co-workers, [1984] showed that the effect of changing the extracellular concentration of magnesium ions was to alter the change in input resistance evoked by NMDA. Magnesium ions apparently blocked the ionophores opened by NMDA thus, giving the response the characteristic increase in input resistance and voltage dependence.

All of these responses could be seen in high magnesium/low calcium containing medium, although those accompanied by a decreased input resistance appeared attenuated, [cf. MacDonald and Wojtowicz,

1982]. In normal recording medium these responses were all accompanied by large increases in the presynaptic activity, both excitatory and inhibitory. Occasionally, L-glutamate responses were apparently biphasic: the first phase of the response was always the depolarization accompanied with a decrease in input resistance. The second phase was either a repolarization which became transiently more hyperpolarized than resting membrane potential or, an NMDA-like prolongation of the depolarization accompanied with a slight increase in input resistance. The transient overshoot in the former response was similar to the after-hyperpolarization which usually accompanied action potentials. A possible reason for this was given by Ransom et al., [1975]; they suggested that there was an increased removal of positive ions from the cell due to an activation of the sodium pump during the prolonged depolarization caused by L-glutamate. The sodium pump being activated by either the high intracellular sodium ion concentration or the low intracellular potassium ion concentration. As the sodium pump exchanges 3 sodium ions for 2 potassium ions [Garrahan and Glynn, 1967], it may be classed as electrogenic. It is this production of an electrical gradient which causes the transient hyperpolarization of the cell. The increase in activity of the sodium pump was only noticable on termination of the L-glutamate response, when the greater efflux of positive ions would shift the membrane potential to a more negative value than resting membrane potential. This effect could be blocked by ouabain [Ransom et al., 1975]. In this thesis the latter of these two responses [the NMDA-like response] could occasionally be evoked by lower doses of L-glutamate, however, a mixture was more common, especially if the cell was bathed in high magnesium concentrations, fig. V.2.

VI.6.2] INHIBITORY AMINO ACIDS

Both the responses evoked by glycine and those evoked by GABA were hyperpolarizations associated with a decrease in membrane resistance. These had similar reversal potentials which were usually close to the resting membrane potential of the impaled neurone. Earlier studies using cultured spinal neurones had reported this type of response to glycine and GABA, [Ransom et al., 1977b; Barker and Ransom, 1978]. As with this study, those authors also reported a dependence of the reversal potential for both responses on the intra- and extra-cellular chloride ion concentration.

The GABA analogue and agonist, EDA [Perkins and Stone, 1982; Blaxter and Cottrell, 1982] evoked no responses from the cultured neurones. However, as reported above, the same neurones readily and repeatedly gave responses to GABA. This ruled out the possibility of desensitization of the EDA/GABA response as a reason for the lack of action. EDA was applied whilst neurones were bathed in normal recording medium. No effect was seen under these conditions. Therefore, this would appear to rule out a possible presynaptic release of GABA by EDA in these cultured spinal neurones. The predominant GABA receptor in the spinal cord is thought to be the GABA_A, which leads to a possible explanation for the inactivity of EDA as it has been reported to have a low potency on GABA_A receptors and act preferentially on a *baclofen*-sensitive receptor, [Perkins and Stone, 1982].

VI.7] AMINO ACID RESPONSES:POSTSYNAPTIC MODULATION BY NEUROPEPTIDES?

Studies on spinal neurones, both in situ and in culture, have mainly been concerned with the activity of opiates [Zieglgansberger and Tulloch, 1979; Nicol et al., 1980; Werz and MacDonald, 1982a]. Similar studies had been performed on cultured neurones with peptides [Barker et al., 1978a; Gruol and Smith, 1981], however, the results of those studies conflicted with each other. As has been reported, responses to the amino acids could be evoked reproducibly from the cultured spinal neurones. Therefore, this preparation appeared perfect for testing the putative postsynaptic modulation of amino acid evoked responses by neuropeptides. As enkephalins appeared to have actions as modulators of amino acid responses, it seemed a reasonable possibility that other neuropeptides may have modulatory activity. For this reason neurotensin, FMRFamide and glycyl L-glutamine were also tested with respect to the amino acids.

No postsynaptic modulatory activity was seen at the concentrations which were usually used to evoke peptide responses from cultured neurones [10 to 200uM]. Occasionally, leu⁵-enkephalin reversibly depressed the glycine response at concentrations in excess of 400uM, when applied by microperfusion. The high concentrations required for this effect lead to the assumption that it was a non-specific action similar to the "strychnine-effect" of morphine reported by Werz and MacDonald, [1982a]. Werz and MacDonald also described the inaction of 20uM D-ala²- met⁵-enkephalinamide on these responses. However, the

dose of opioid peptide was not comparable to that of opiate alkaloid. The effect of leu⁵-enkephalin reported by Barker and co-workers, [1978a] showed that at high iontophoretic doses this peptide may also antagonise the action of glycine.

Werz and MacDonald, [1982a] also reported an effect of morphine on the GABA response. This effect was not shown in this thesis possibly because of the much greater concentration of opioid peptide which would be required to produce it.

It would appear that the neuropeptides do not postsynaptically modulate amino acid evoked responses, in these cultured spinal neurones. It was possible that the sample size used in this study may have precluded the finding of any changes in response amplitude; especially if only a small percentage of the neurones could show these effects. However, this does not appear to be the case from the reports of Barker and co-workers [1978a], who found this type of response on over 50% of their cultured spinal neurones.

VI.8] CONCLUSIONS

Spinal neurones from the mouse embryo were successfully prepared in a primary dissociated cell culture and could be maintained for in excess of 10 weeks. The control of contamination was a priority because antibiotics and fungicides were not used. However, these problems were minimized by refining techniques as well as continual care and attention.

Intracellular recordings made from the spinal neurones showed

they were viable. The morphology, electrophysiology and chemosensitivity to amino acids [GABA, glycine and L-glutamate] confirmed previously reported studies. However, few neurones were found which showed sensitivity to the peptides. Further to this, there was a great variation in the number of responsive neurones found in each batch of cultures. This led to the conclusion that some tissue, which presumably supplied a "growth factor", was being occasionally included [or omitted] during the dissection. To investigate this possibility, other tissues and organs from the mouse embryo were included with the spinal cords in culture. The percentage of neurones which responded to peptides from the co-cultures, however, was no higher than the corresponding percentage from pure spinal cord cultures.

A variety of peptide responses were found. Of particular significance were those responses to FMRFamide which were the first reported from intracellular recordings from mammalian neurones. These direct responses to FMRFamide imply that this compound may have an active counterpart in the mammalian CNS. Furthermore this counterpart may have a role as a modulator of neuronal activity in the mammalian spinal cord. The recently discovered dipeptide, glycyl L-glutamine, was another peptide of which this was the first intracellular study in the mammalian CNS. The inhibitory responses found in this study confirmed and extended the work of Parish et al., [1983] in rat brainstem. This leads to the possibility of a neurohumoral role for this peptide which, although not currently known to be present in the mammalian spinal cord, may be cleaved from B-endorphin in the cerebrospinal fluid to act in the spinal cord.

This thesis has described a possible inhibition of neuronal activity by neurotensin. However, this contrasts with the only other intracellular study of the mammalian spinal cord [Stanzione and Zieglgansberger, 1983]. It is possible that the neurones responsible for these responses were from an area of the spinal cord other than the substantia gelatinosa. However, further studies in the intact spinal cord would be necessary to verify this. The responses evoked from the spinal neurones by enkephalin appeared similar to those mentioned in a previous study by Barker and co-workers, [1978a]. However, in this thesis only direct "neurotransmitter-like" responses were found. Apart from those responses which would be classed as inhibitory [the expected action of the opioids], a small number of excitatory responses were seen. These have not been shown previously with enkephalin and were unlike most of the other responses evoked from these neurones.

An investigation was made into the possible modulation of amino acid responses by the peptides. This study was performed on spinal neurones which had previously given no measurable response to the peptide. The results obtained showed no interaction or modulation of the amino acid responses from these spinal neurones. In the case of glycyl L-glutamine, the structural similarity between it and certain of the L-glutamate antagonists was tested by a study of the L-glutamate response before and in the presence of this peptide. No antagonism was found between the peptide and L-glutamate.

The studies reported in this thesis provide evidence for the existence of many types of peptide evoked response which have not yet been found in intact tissue. This is possibly because of two

reasons: the small number of responsive neurones and that those neurones are located in areas of the spinal cord where the low density of peptide receptors would make any study extremely tedious.

Further research aimed at extending these studies can move in two directions. An investigation of the underlying mechanisms would best be performed in a dissociated cell culture. Problems associated with the low number of responsive neurones present in such a culture may soon be overcome with the help of cell sorting systems. The monocultures produced would allow in depth studies not only with single or double electrode voltage clamp, but also with patch clamp techniques. The other direction would lead to a study into the location of these peptide responses in the intact spinal cord. This would help elucidate the physiological relevance of the response and in what way the response [and therefore the peptide] interacts within the neuronal matrix which is the spinal cord.

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